

**STUDIES OF SEROLOGY AND VASCULAR ULTRASOUND IN PATIENTS WITH SYSTEMIC LUPUS
ERYTHEMATOSUS – FOCUS ON CARDIOVASCULAR DISEASE**

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“Para ser grande, sê inteiro: nada

Teu exagera ou exclui.

Sê todo em cada em cada coisa. Põe quanto és

No mínimo que fazes.

Assim em cada lago a lua toda

Brilha, porque alta vive.”

Ricardo Reis/ Fernando Pessoa, 1888 – 1935

*(To be great, be whole: nothing of yours exaggerate or exclude.
Be entire in everything. Give all of you in the least you do.
Hence on every lake the whole moon shines as it lives high”)*

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DECLARATION OF WORK

I confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Sara Jesus Cameira Croca

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ABBREVIATIONS

- ❖ 2D: two-dimension
- ❖ 37°C: 37 degrees Celsius
- ❖ 3D: three-dimension
- ❖ 3-NTy: 3-nitrotyrosine
- ❖ AC: Afro-Caribbean origin
- ❖ ACE: angiotensin-converting enzyme
- ❖ ACR: American college of Rheumatology
- ❖ ACS: acute coronary syndrome
- ❖ AHA: American Heart Association
- ❖ ANA: antinuclear antibodies
- ❖ Anti-ApoA1: anti-apolipoprotein 1 antibodies
- ❖ Anti-dsDNA: anti-double stranded DNA antibodies
- ❖ Anti-NCS: anti-nucleosome antibodies
- ❖ ARDS: acute respiratory distress syndrome
- ❖ ATP-binding cassette transporter A1: ABCA1
- ❖ AU: absorbance units
- ❖ BILAG: British Isles Lupus Assessment Group index
- ❖ BSA: bovine serum albumin
- ❖ C: Caucasian origin
- ❖ C3: complement fraction 3
- ❖ CAD: coronary artery disease
- ❖ CaM: calmoduline
- ❖ CCA: common carotid artery
- ❖ CFA: common femoral artery
- ❖ CETP: cholesteryl ester transfer protein
- ❖ CNS: central nervous system
- ❖ CRP: C-reactive protein
- ❖ CT: computed tomography
- ❖ CVD: Cardiovascular disease
- ❖ Cys: cysteine
- ❖ D-HDL: dysfunctional HDL
- ❖ EBV: Epstein-Barr virus
- ❖ ECA: external carotid artery
- ❖ ENA: anti-extractable nuclear antigens antibodies

- ❖ FGF 2: fibroblast growth factor 2
- ❖ HbA_{1c}: Glycated haemoglobin
- ❖ GSM: grey scale median
- ❖ h: hour
- ❖ HDL: high-density lipoprotein
- ❖ HIF: hypoxia-inducible transcription factor
- ❖ HQ: hydroxychloroquine
- ❖ HRP: horse radish peroxidase
- ❖ HRT: hormone replacement therapy
- ❖ ICA: internal carotid artery
- ❖ ICAM 1: intercellular adhesion molecule 1
- ❖ IDL: intermediate-density lipoprotein
- ❖ IFN α : interferon alpha
- ❖ IL1: interleukin 1
- ❖ IMT: intima-media thickness
- ❖ IQR: interquartile range
- ❖ IVUS: intra vascular ultrasound
- ❖ Lp: lipoprotein
- ❖ LDL: low-density lipoprotein
- ❖ lecithin-cholesterol acetyltransferase: LCAT
- ❖ MGv: mean grey value
- ❖ MHC: major histocompatibility complex
- ❖ MI: myocardial infarction
- ❖ min: minutes
- ❖ MRI: magnetic resonance imaging
- ❖ NA: nitrated albumin
- ❖ Nitrated ApoA1: nitrated apolipoprotein A1
- ❖ NN: nitrated nucleosomes
- ❖ NO: nitric oxide
- ❖ NOS: nitric oxide synthetase
- ❖ NSAIDs: non-steroidal anti-inflammatory drugs
- ❖ OD: optical density
- ❖ paraoxonase 1: PON1
- ❖ PBS: phosphate buffer solution
- ❖ PC: positive control
- ❖ PDGF: platelet-derived growth factor
- ❖ RA: rheumatoid arthritis

- ❖ RNS :reactive nitrogen species
- ❖ RNS: reactive nitrogen species
- ❖ ROS: reactive oxygen species
- ❖ ROS: reactive oxygen species
- ❖ RT: room temperature
- ❖ SLE: Systemic Lupus Erythematosus
- ❖ SLICC: Systemic Lupus International Collaborative Clinics
- ❖ TCFA: thin-cap fibroatheroma
- ❖ t-cholesterol: total cholesterol
- ❖ TF: tissue factor
- ❖ TG: triglycerides
- ❖ TGF b: transforming growth factor b
- ❖ TNF α : tumour necrosis factor alpha
- ❖ TpA: Total plaque area (mm²)
- ❖ TpT: Total plaque thickness (mm)
- ❖ Ty: tyrosine
- ❖ UCLH: University College London Hospital
- ❖ US: ultrasound
- ❖ UV: ultraviolet
- ❖ VLDL: very low-density lipoprotein
- ❖ VEGF: vascular endothelial growth factor

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ABSTRACT

Systemic lupus erythematosus (SLE) is a multisystem, autoimmune disease characterised by the presence of autoantibodies. Active inflammation in patients with SLE can cause clinical effects in various organs and serum biomarkers can be helpful in monitoring the disease. Over time, patients with SLE have an increased risk of developing atherosclerosis. This risk is partially due to standard risk factors such as smoking and hypertension, but also due to SLE-specific immunological factors. In this thesis I have studied three linked questions;

1) Systemic inflammation can lead to endothelial activation and increased release of nitric oxide. This leads to nitration of serum proteins. Using capture ELISA, I studied levels of nitrated nucleosomes and nitrated albumin in patients with SLE. Nitrated nucleosome levels were higher in patients with SLE than controls, and were related to some forms of disease activity notably in the central nervous system, but not atherosclerosis.

2) High-density lipoprotein (HDL) and its main structural component, apolipoprotein A1 (ApoA1), have an atheroprotective role. Using direct ELISA, I measured serum levels of anti-ApoA1 and anti-HDL antibodies in patients with SLE. IgG anti-apoA1 levels were associated with increased disease activity and with drug treatment, but not with atherosclerosis.

3) Previous authors used vascular ultrasound to demonstrate asymptomatic carotid plaque in between 30% and 40% of patients with SLE. I used new, more sensitive measures of carotid and femoral atherosclerosis in 100 patients with no previous history of CVD to investigate the factors associated with presence, area, volume and echogenicity of plaque. Thirty-six patients had plaque. Intima-media thickness (IMT) was associated with age and disease duration but not activity. Total plaque area (TPA), however, was increased in patients with persistently active disease and also with anti-HDL levels. Lipid profile was associated with both IMT and TPA. Plaque echogenicity was associated with disease activity and renal function. Comparison of these 100 SLE patients with an age/sex matched control group showed significant differences in morphology and echogenicity of plaque.

1: INTRODUCTION

1.1: Systemic Lupus Erythematosus

1.1.1: Pathogenesis

Systemic lupus erythematosus (SLE) is a chronic, autoimmune, multisystem disease whose hallmark is the heterogeneity of its clinical presentations and the unpredictability of disease flares and remissions (Merrill and Buyon 2005, Rahman and Isenberg 2008, Ruperto, Hanrahan et al. 2011). The various clinical phenotypes result from a complex interplay between immunological (C3, anti-dsDNA), genetic and environmental factors which culminates with an irreversible break in immunological tolerance (Isenberg 1997).

Environmental triggers include viral infections, drugs and ultraviolet (UV) light. The virus most commonly associated with the onset of lupus or flares is EBV. EBV-antibodies and EBV DNA have been reported in 99% and 100% of children and young adults with SLE (James, Kaufman et al. 1997). SLE-like features may be induced by several drugs, namely hydralazine, procainamide and quinidine and are usually limited to the skin and joints. The environmental factor whose link to SLE is best established is UV radiation and the occurrence of a photosensitive rash is included in the American College of Rheumatology revised (ACR) classification criteria for SLE (Tan, Cohen et al. 1982, Hochberg 1997).

The relevance of the hormone environment, namely sexual hormones, in the pathogenesis of lupus is well illustrated by the marked female predominance (10:1) but the exact mechanisms remain unknown.

Genetics also appear to be pivotal in the pathogenesis of SLE: concordance rates for lupus are 25% for monozygotic twins compared with 2% for dizygotic twins. Other genetic factors have been identified, namely certain HLA subtypes (A1, B8 and DR3) and null alleles associated with complement (C1q, C2 or C4) (Rahman and Isenberg 2008). These genes encode components of the immune system. HLA is part of the major histocompatibility complex (MHC) which mediates the interaction between antigen and antigen-presenting cell which in turn triggers the activation of T-cells. Complement activation is a key element in immune-complexes-mediated response found in SLE which is thought to be responsible to a great extent for target-organ damage, namely in pathogenesis of lupus nephritis. Epigenetic changes namely DNA methylation and post-translational modification of histones either inherited or induced by environmental factors are also thought to play a role (Wallace and Hahn 2007).

The pivotal trigger in the development of SLE is the presence of an immune response against endogenous nuclear antigens. Auto-antigens are likely to derive from apoptotic debris whose clearance is thought to be impaired. The presence of large amounts of circulating endogenous nucleic acids leads to interferon alpha ($IFN\alpha$) production, which in turn stimulates antigen-presenting cells to present them. Consequently, T cells and B cells are activated, ultimately leading to the mounting of a full-blown immunological response against self-antigens. The consequent antibody and inflammatory cytokine production provide the necessary stimulus for the perpetuation of this process, namely through an immune-complex mediated response (Wallace and Hahn 2007).

Immune complexes and complement activation are thought to be the main mediators of target-organ damage. Failure to clear immune complexes allows them to deposit in tissues leading to the recruitment and activation of inflammatory cells such as neutrophils and macrophages. The production of inflammatory cytokines such as tumour necrosis factor alpha ($TNF\alpha$) and reactive oxygen species (ROS) ultimately leads to tissue injury and target-organ dysfunction (Wallace and Hahn 2007). Complement depletion/ consumption as a result of this process are other characteristic serological features of SLE.

More than 100 autoantibodies have been identified in relation with SLE, the majority of which are not relevant in clinical practice (Sherer, Gorstein et al. 2004) and I shall therefore focus on those that are.

Anti-nuclear antibodies (ANA) are the most commonly identified antibodies in lupus and although the specificity of this test is low (10-35% of healthy individuals >65 years are ANA positive), its sensitivity is high: ANA negative patients have a less than a 3% chance of having SLE. The titres of ANA vary, but they do not appear to correlate with disease activity or organ-specific involvement. Contrary to ANA, anti-double stranded DNA antibodies (anti-dsDNA) (Rahman and Isenberg 2008) are highly specific for SLE: they are present in 70% of patients with lupus and less than 0.5% of healthy controls and their relevance in the pathogenesis of SLE is well established (Isenberg, Manson et al. 2007). In addition, serum levels of anti-dsDNA appear to reflect disease activity in the majority of patients (ter Borg, Horst et al. 1990). A small number of patients have high anti-ds-DNA and/ or low C3 levels with no clinical evidence of disease activity i.e., although they appear to be serologically active they remain clinically quiescent (Ng, Manson et al. 2006). However, 80% of these patients suffer a disease flare within a 5-year period (Ng, Manson et al. 2006, Rahman and Isenberg 2008).

Another group of autoantibodies found in SLE are the anti-extractable nuclear antigens antibodies (ENA) which are a heterogeneous group of antibodies whose targets are non-DNA antigens. ENA levels tend not to vary over time (Agarwal, Harper et al. 2009). Moreover, they don't appear to be affected by the use of B cell depletion and it is thought they are produced by long-lived plasma cells (Ng, Leandro et al. 2006). There are four main ENA subtypes: anti-Ro, anti-La, anti-Sm and anti-RNP. Anti-Ro and anti-La antibodies are associated with an increased risk for foetal congenital heart block (Costedoat-Chalumeau, Amoura et al. 2005, Brucato 2008). Anti-Ro and anti-La also appear to be associated with sicca syndrome, skin involvement and photosensitivity (Cervera, Khamashta et al. 1993). Anti-Sm has a relatively low prevalence (10-30%), but it is the most specific antibody for SLE and its presence is considered pathognomonic (Mannik, Merrill et al. 2003, Ng, Leandro et al. 2006, Wallace and Hahn 2007). Anti-RNP antibodies are associated with 'overlap features' including Raynaud's phenomenon and myositis.

Finally, anti-nucleosome antibodies (anti-NCS) have been found in the sera of 70-90% patients with lupus and their levels were found to correlate with disease activity, namely with renal flares (Amoura, Koutouzov et al. 2000, Ng, Manson et al. 2006, Muller, Dieker et al. 2008). In addition, it appears that patients with high anti-NCS levels tend to flare more frequently. Despite this, anti-NCS levels are not used routinely in clinical practice and this is probably due to the need for a laborious technique and insufficient added benefit to other serological markers such as C3 and anti-dsDNA.

1.1.2: Epidemiology and clinical Features

The overall prevalence of SLE ranges between 40 to 200 per 100,000 persons depending on the predominant ethnic origin considered (Rahman and Isenberg 2008). Epidemiologic studies reflecting different ethnic backgrounds suggest that SLE is more frequent in non-Caucasian individuals (Danchenko, Satia et al. 2006, Lau, Yin et al. 2006, Jakes, Bae et al. 2012). Ethnicity also appears to influence the type of SLE presentation. In particular, AC-origin patients frequently present with severe renal disease and hypertension (Ward and Studenski 1990), while Hispanic-origin patients have a greater incidence of arthritis (Vila, Alarcon et al. 2004). Although these differences suggest that the genetic background could play an important role, the impact of environmental factors, namely socio-economic, must also be taken into consideration (Murphy and Isenberg 2013).

A clear female predominance is observed in SLE with over 90% of patients being women (Rahman and Isenberg 2008), which suggests a central role for sex hormones in the

pathogenesis of the disease. This is supported by several studies which showed an association between the use of exogenous hormones (contraceptive pill and hormone replacement therapy – HRT) with lupus onset and flares (Costenbader, Feskanich et al. 2007). Furthermore, a correlation between age and incidence of SLE mirrors peak years of female sex hormone production, with a peak during childbearing years (Manzi, Wallace et al. 2001). The onset of SLE occurs usually after puberty, typically between the 20s and 30s, with the highest prevalence of SLE being amongst women aged between 14-64 years (Manzi, Wallace et al. 2001). Finally, the female to male ratio tends to decrease with age, with a fall in prevalence among women which remains stable throughout in men (Boddaert, Huong et al. 2004). There are various target organs potentially involved in SLE, most commonly joints, skin and blood (>80% patients) but also the kidneys, central nervous system (CNS) and cardiopulmonary systems (30-50%) (Dean, Tyrrell-Price et al. 2000). In addition to organ specific symptoms, systemic manifestations such as fatigue, malaise, fever and anorexia can also occur. The first criteria for SLE classification were established in 1971 and later revised in 1982 (Tan, Cohen et al. 1982) and 1997 (Hochberg 1997) by the American College of Rheumatology. The latest version is showed in Table 1. An alternative classification was proposed by the Systemic Lupus International Collaborating Clinics (SLICC) in 2012 (Petri, Orbai et al. 2012). Seventeen classification criteria derived from wide a set of expert-rated patient scenarios were considered, depicting a wide range of multi-organ clinical features and immunological findings. The SLICC criteria for SLE classification requires: 1) Fulfilment of at least four criteria, with at least one clinical criterion AND one immunologic criterion OR 2) Lupus nephritis as the sole clinical criterion in the presence of ANA or anti-dsDNA antibodies. In the derivation set, the SLICC classification criteria resulted in fewer misclassifications than the current ACR classification criteria (49 versus 70, $p=0.0082$), had greater sensitivity (94% versus 86%, $p<0.0001$) and equal specificity (92% versus 93%, $p=0.39$). In the validation set, the SLICC Classification criteria resulted in fewer misclassifications (62 versus 74, $p=0.24$), had greater sensitivity (97% versus 83%, $p<0.0001$) but less specificity (84% versus 96%, $p<0.0001$). Amezcua-Guerra et al compared the 1997 ACR and the 2012 SLICC criteria and concluded that both systems were similar to classify SLE in an uncontrolled real-life scenario with the caveat that several new items contained in the 2012 SLICC criteria could represent a step forward for research purposes in selected clinical settings (Amezcua-Guerra, Higuera-Ortiz et al. 2015).

Criteria	Definition
Malar rash	Fixed erythema, flat or raised, over the malar eminences, tending to spare the nasolabial folds.
Discoid rash	Erythematous raised patches with adherent keratotic scaling and follicular plugging. Atrophic scarring may occur in older lesions.
Photosensitivity	Skin rash as a result of an unusual reaction to sun light by patient history or physician observation.
Oral ulcers	Oral or nasopharyngeal ulceration, usually painless, observed by physician.
Non-erosive Arthritis	Involving 2 or more peripheral joints, characterized by tenderness, swelling or effusion.
Serositis	Pleuritis: convincing history of pleuritic chest pain or rubbing heard by a physician or evidence of pleural effusion. <u>OR</u> Pericarditis: documented by electrocardiogram, rub or evidence of pericardial effusion.
Renal disorder	Persistent proteinuria ($>0.5\text{g/day}$ or $>3+$ if quantitation not performed) <u>OR</u> Cellular casts (red cells, haemoglobin, granular, tubular or mixed)
Neurologic disorder	Seizures (in the absence of other precipitating factors) <u>OR</u> Psychosis (in the absence of other precipitating factors)
Hematologic disorder	Haemolytic anaemia with reticulocytosis <u>OR</u> Leukopenia ($<4,000/\text{mm}^3$ on ≥ 2 occasions) <u>OR</u> Lymphopenia ($<1,500/\text{mm}^3$ on ≥ 2 occasions) <u>OR</u> Thrombocytopenia ($<100,000/\text{mm}^3$ in the absence of other precipitating factors)
Immunologic Disorder	Anti-dsDNA <u>OR</u> Anti-Sm <u>OR</u> Anti-Phospholipid antibodies (IgG/ IgM anticardiolipin antibodies or positive lupus anticoagulant using a standard method or false-positive test result for at least 6 months confirmed by <i>Treponema pallidum</i> immobilization or fluorescent treponemal antibody absorption test).
Positive ANA	Abnormal titre of ANA by immunofluorescence or and equivalent assay at any time point in time and in the absence of precipitating factors (drugs)

Table 1: Update of the 1982 ACR Revised Criteria for the Classification of SLE (adapted from Hochberg et al).

A patient may be classified as having SLE when at least 4 criteria are present, although they do not necessarily have to be present simultaneously. These classification criteria show excellent sensitivity and specificity (>85% and >95% respectively) in established disease and were developed mainly to be used in clinical studies as a way to ensure that the literature is uniform when referring to SLE.

In addition to the signs and symptoms included in the classification criteria, there is a much broader range of clinical features which can be found more or less frequently in patients with SLE which correlate with the involvement of specific target organs, such as the skin, musculoskeletal system, kidney and nervous system.

There is a growing awareness of the importance of co-morbidities related directly with SLE or associated with treatment, namely the high incidence of cardiovascular disease which remains one of the main causes of mortality and morbidity for patients with SLE (CVD-related morbidity will be discussed at length on section 1.2.5: Cardiovascular disease and SLE). Other co-morbidities such as vitamin D deficiency and osteoporosis have a high incidence among patients with SLE due to the chronic use of steroid-based treatment regimens, renal disease and avoidance of sun exposure. The prevalence of osteopenia can reach as high as 75%, while osteoporosis is reported in over half the patients (Bichile and Petri 2014). The potential increased occurrence of malignancy was also a concern due particularly to the use of immunosuppressants such as cyclophosphamide. An increased risk of haematological malignancies such as non-Hodgkin's lymphoma has been a concern and most recently, the standardized incidence ratio was raised 3-fold in patients with SLE (Bernatsky, Ramsey-Goldman et al. 2013) but this did not reach statistical significance. Other malignancies such as lung cancer, hepatocellular carcinoma and genital cancers may also be more prevalent in SLE (Ramsey-Goldman, Mattai et al. 1998). The use of immunosuppressive drugs may impair viral clearance and eradication which could explain the excess of human papilloma virus-associated cancers among women in SLE (Bernatsky, Joseph et al. 2008).

1.1.3: Treatment and Prognosis

SLE is characterized by the presence of chronic inflammation with periods of exacerbation alternating with sustained low grade activity which can be clinically non-apparent and even quiescent disease. Regardless of the degree of systemic inflammation and clinical manifestations, the presence of a largely chronic inflammatory state may result in permanent organ damage. Controlling inflammation is therefore of paramount importance when treating

patients with SLE. The first step on the treatment ladder came in the early 50's, following Philip Hench's use of compound E (hydrocortisone) in patients with inflammatory arthritis (Luijten, Fritsch-Stork et al. 2013). Since then, the cornerstone of lupus-therapy has been the use of glucocorticoids with a major improvement on survival: from a 4-year survival rate of only 50% in the 50's, to 80% of patients being alive 15 years after being diagnosed with SLE nowadays (Isenberg and Rahman 2006).

However, the efficacy of steroid-based regimens comes with a considerable array of side effects, including hypertension, osteoporosis, glucose and lipid metabolism impairment. In order to achieve optimal disease activity control while minimizing steroid dose requires the use of steroid sparing agents. The most commonly used steroid sparing drugs are immunosuppressive agents like methotrexate (MTX), azathioprine (AZA), mycophenolate mofetil (MMF) and cyclophosphamide (CYC). CYC is a powerful cytostatic alkylating agent usually reserved for severe manifestations and until recently was considered the standard care for proliferative lupus nephritis, usually in combination with pulsed corticosteroids on a monthly basis until disease control is achieved (Illei, Austin et al. 2001). Despite being a highly effective drug, CYC is associated with significant toxicity and side effects ranging from an increased risk of infections, infertility and increased risk of malignancies (Radis, Kahl et al. 1995, Wang, Wang et al. 1995). Several alternative immunosuppressive agents have therefore been considered and used to limit the need to prescribe CYC. MTX is a folic acid analogue which inhibits purine synthesis and adenosine deaminase activity used mainly to treat joint and skin manifestations of SLE but its efficacy is modest when major organ system involvement is concerned (Fortin, Abrahamowicz et al. 2008). MMF and AZA are immunosuppressants which act as DNA-synthesis inhibitors which are usually reserved for major organ involvement such as nephritis (Contreras, Pardo et al. 2004). MMF appears to be more effective than AZA as an induction treatment for lupus nephritis and some studies even suggest superiority compared with CYC in induction therapy (Ginzler, Dooley et al. 2005). Moreover, subgroup analysis appears to favour the use of MMF among patients of African American and Hispanic descent (Isenberg, Appel et al. 2010). In addition, MMF is a direct active agent whereas AZA must be metabolized into its active metabolite 6-mercaptopurine which is predominantly cleared by thiopurine methyltransferase. Polymorphisms affecting this enzyme activity are notoriously common and can lead to increased toxicity, including myelosuppression and hepatotoxicity (Sahasranaman, Howard et al. 2008). Nonetheless, the use of AZA as a steroid-sparing agent in maintenance regimens is both effective and safe (Contreras, Pardo et al. 2004, Mok, Ying et al. 2009).

Another drug class which is both effective and safe in the lupus treatment armoury are the anti-malarial agents like hydroxychloroquine. Their mechanism of action is not yet fully

understood but it is thought that they affect leukocyte phagocytosis and migration through inhibition of lysosome acidification (Kuznik, Bencina et al. 2011). They also affect binding to Toll like Receptors [TLR 7 and 9]. Antimalarial agents have a good safety profile with retinopathy being a rare complication. It has been shown that its use limits disease flares and decreases accrual damage particularly when started early in the disease course (Group 1991, James, Kim-Howard et al. 2007). Further beneficial effects on lipid profile and anti-thrombotic properties have also been recognized as discussed on section 1.2.5: Cardiovascular Disease and SLE.

Further improvement can be achieved through the better management of complications such as infections (antibiotics) and renal disease (angiotensin converting enzyme (ACE)-inhibitors).

Due to treatment optimization and, perhaps no less importantly, earlier diagnosis, survival has improved dramatically. However, the bimodal pattern of mortality in SLE first described by Urowitz *et al.* (Urowitz, Bookman et al. 1976) is still observed. Early mortality occurring in the first 5 years after diagnosis is mainly due to infection and severe renal disease, whereas late deaths occurring in patients with relatively inactive disease were mainly attributable to cardiovascular disease (CVD). This pattern has been repeatedly observed by other groups and although mortality and morbidity improvement has been achieved, the rate of CVD-related complications remains high (Rubin, Urowitz et al. 1985, Frostegard 2008, Croca, Rodrigues et al. 2011).

In fact, despite all the advances made, prolonged, complete remission remains elusive for the majority of patients. One way to improve prognosis may be through earlier diagnosis of complications usually associated with late mortality. The development of better ways to identify CVD, ideally before symptomatic disease manifests, could potentially have a positive impact in reducing mortality and morbidity in patients with SLE.

Over recent years, the use of targeted therapies focused mainly on B cells but also on specific inflammatory cytokines and pathways has been explored. Blocking IL 6 and IFN α have generated increasing interest, as have therapies targeting costimulatory interactions and small molecule inhibitors that interfere with cell signalling pathways (Lo and Tsokos 2012, Sthoeger, Sharabi et al. 2014). Nonetheless, despite all the new agents targeting different aspects of the inflammatory response, the anti-B-cell biologics have been shown to yield the most consistent benefits in terms of achieving optimal disease control while minimizing steroid and immunosuppression use. Either through B cell depletion agents such as rituximab and ocrelizumab or by antagonizing the effects of B lymphocyte stimulator factors (belimumab), the use of targeted biological therapy is gradually becoming more

appealing as it promises to be effective and safe, with less toxicity and lower risk of infections compared with the classic treatment regimens. This shift in the treatment paradigm is best illustrated by the approval of belimumab as the first novel therapy for SLE in over 50 years. Multicentre, randomized controlled trials with long term follow up are required to prove not only the efficacy but also the safety and cost-effectiveness of these new drugs compared with the current standard of care (Cobo-Ibanez, Loza-Santamaria et al. 2014, Kamal 2014).

1.1.4: Disease Activity Definition –BILAG Scoring System

When assessing patients with SLE, generally three patterns of disease activity can be identified: disease flare, chronically active disease and quiescence (Nuttall and Isenberg 2013). Disease activity may be defined based on clinical features and/ or serological markers such as anti-dsDNA and C3. Importantly, clinical aspects attributable to active SLE must be distinguished from chronic damage or non-related causes namely infections and drug toxicity (Feld and Isenberg 2014). Defining disease activity in SLE is of paramount importance as it is the basis for majority of treatment decisions. Moreover, it is also crucial for the design of clinical trials, an improvement in disease activity must be one of the most relevant end-points considered. Serologically, raised anti-dsDNA levels in combination with low C3 levels are usually an indication of underlying active disease. However, serology alone is insufficient to assess the severity and extent of a disease flare and therefore, several clinical activity indices have been developed over the years (Bombardier, Gladman et al. 1992, Urowitz and Gladman 1998, Isenberg and Ramsey-Goldman 1999, Strand, Gladman et al. 1999). The most commonly used are the British Isles Lupus Assessment Group Scale (BILAG), the SLE Disease Activity Index (SLEDAI), the European Consensus Lupus Activity Measure (ECLAM) and the Systemic Lupus Activity Measure (SLAM). These indices were based on long term observational studies and have been shown to reflect changes in disease activity as well as predict damage and mortality. The BILAG differs from the other lupus activity indices because rather than providing a global score for disease activity, it reports disease activity in different organs/ systems separately (Symmons, Coppock et al. 1988, Hay, Bacon et al. 1993, Gordon, Sutcliffe et al. 2003). Nonetheless, there is a positive correlation between all these indices and presently there is no indication of the superiority of one over the others. The scoring system routinely used at the Lupus Clinic at University College London Hospital (UCLH) is the BILAG and therefore, for the purpose of my thesis I will only focus on this index.

The BILAG was developed with the aim of providing accurate and reliable activity assessment across a range of organs and systems. It is based on the principle of the physician's 'intention to treat'. (Gordon, Sutcliffe et al. 2003). To score on the index, the symptom/ sign must have been present within the previous 4 weeks and it has to be unequivocally ascribable to SLE rather than to a non-specific intercurrent condition such as an infection.

In the original BILAG there are eight organ or systems considered: general, mucocutaneous, neurological, musculoskeletal, cardiorespiratory, vasculitis, renal and haematological. A score is given to each system based on the clinical features present over the last 4 weeks depending upon whether they are new, worse, the same or improving compared to previously (Gordon, Sutcliffe et al. 2003). Although the score can be calculated manually, a purpose designed software has been developed (British Lupus Integrated Prospective System – BLIPS (Isenberg and Gordon 2000)). Immunological data do not contribute to the BILAG score, although basic haematology and renal function assessment help to determine the scores of the respective systems.

Each organ system receives a score between A and E. An "A" score denotes severely active disease requiring disease-modifying treatment i.e. high dose steroids (prednisolone >20mg/day) and/or immunosuppressants. A score of "B" signifies that although the disease is active, the physician would only consider treating it with NSAIDs, hydroxychloroquine or less than 20mg prednisolone daily. "C" describes stable mild disease and "D" refers to a system which is currently inactive but which had previously been affected. Finally, a score of "E" means that that particular system was never involved. A severe flare of lupus can be defined as a new score of A in any system and a moderate flare has been defined as a score of B in any system which previously scored D or E (Gordon, Sutcliffe et al. 2003).

A numerical score can also be derived from the BILAG providing a global disease activity score which correlates well with the global scores from the SLEDAI, SLAM and ECLAM. A global BILAG score was initially designed to be calculated from the formula A=9; B=3; C=1; D=0 and E=0 (Stoll, Stucki et al. 1997). More recently, a revised optimal scoring system for classic BILAG has been published in which A= 12; B= 5; C= 1; D and E=0 (Yee, Cresswell et al. 2010). Unless stated otherwise, whenever I mention the global BILAG score I will be referring to this later numerical version.

During the last few years new version of the BILAG (BILAG 2004) was produced. However, most of the serum samples that I used in my longitudinal studies were taken before BILAG 2004 was available, so that in thesis I refer to the classic BILAG score unless otherwise stated.

1.2: Cardiovascular Disease

1.2.1: Atherosclerosis – Pathophysiology

Currently, it is thought that atherosclerosis is essentially a chronic inflammatory process (Ross 1999) leading to the transition from an incipient fatty streak to a mature atheromatous plaque. The fatty streak, which is common in children and young infants, is the earliest type of lesion and is purely inflammatory, consisting only of monocyte-derived macrophages and T lymphocytes. It is hypothesized that the premise for atherosclerosis is a response-to-injury mechanism, where endothelial dysfunction plays the initial event which triggers the subsequent events. Potential causes for endothelial dysfunction include raised and modified lipids (oxidized LDL), free radicals caused by smoking, hypertension, diabetes, elevation of plasma homocysteine levels and infections (*Chlamydia pneumonia*). All of these and other factors combined with a predisposing genetic background lead to endothelial damage and ultimately to endothelial dysfunction and atherosclerosis (Ross 1999). Following endothelial dysfunction, several compensatory responses arise which in turn change the normal homeostatic properties of the endothelium. There is an increased endothelial permeability mediated mainly by nitric oxide (NO), prostacyclin, platelet-derived growth factor (PDGF) and angiotensin II as well as an increased adhesiveness, mainly mediated by L-selectin, E-selectin, P-selectin and intercellular adhesion molecule 1 (ICAM1). Furthermore, there is a loss of the normal anticoagulant nature of the endothelium which develops procoagulant properties through the production of vasoactive molecules, cytokines and growth factors. If this process continues uninterrupted, smooth muscle cells proliferate and migrate through the action of various cytokines such as PDGF, fibroblast growth factor 2 (FGF2) and transforming growth factor beta (TGF β), forming an intermediate lesion. This process called remodelling eventually leads to arterial wall thickening and to a compensatory arterial dilatation aimed at preserving the arterial lumen. At this stage, inflammatory cells (mainly monocyte-derived macrophages and T lymphocytes) adhere to the endothelial surface and enter the arterial wall (diapedesis), multiplying within the lesion. Their activation leads to the release of proteolytic enzymes, cytokines, chemokines and growth factors ultimately producing focal necrosis. In later stages, neutrophils are also recruited. Their main effects can be divided into two groups: proteolytic (leading to gradual thinning of the fibrous cap) and oxidising, through the production of ROS which contribute to the modification of lipids present in the plaque.

Moreover, circulating lipids, mainly low density lipoproteins (LDL), start to seep into the arterial wall where they undergo progressive oxidation, ultimately stimulating scavenger receptors in the surface of macrophages and leading to the phagocytosis of oxidized-LDL.

However, the internalization of LDL by macrophages leads to the formation of lipid peroxides and facilitates the accumulation of cholesterol esters, resulting in the formation of foam cells. The formation of foam cells leads to the removal and sequestration of modified LDL, minimizing their effects on the endothelial and smooth muscle cells. However, the ability of macrophages to sequester LDL is limited and once their capacity is surpassed, the accumulation of modified LDL within the arterial wall is inevitable. In addition to the effects previously described, oxidised LDL also has a chemotactic effect, amplifying the recruitment of inflammatory cells. Additionally, oxidised LDL induces the production of inflammatory cytokines such as $\text{TNF}\alpha$ and interleukin 1 (IL1) which in turn enhance the binding of LDL to the endothelium and smooth muscle cells. Platelets also play a pivotal role through signalling pathways bridging inflammation and thrombosis (Croce and Libby 2007). In response to injury, the endothelium releases von Willebrand factor which leads to platelet recruitment to the plaque site. The recruited platelets then bind to the endothelial cells via an interaction between glycoprotein I and von Willebrand factor. Furthermore, this interaction leads to platelet activation and production of vasoactive molecules such as thromboxane A₂, adenosine and thrombin as well as other proinflammatory mediators like PDGF which in turn are involved in the recruitment and activation of inflammatory cells.

Finally, another contributor for atherothrombosis is the formation of new blood vessels. It is thought that the main trigger for angiogenesis is the hypoxic environment within the arterial wall which leads to the production of vascular endothelium growth factor (VEGF) and hypoxia inducible factors (HIF). The new vessels formed possess leaky endothelial junctions and contribute to an increased risk of haemorrhage within the plaque. The end result of repeated cycles of inflammatory cells recruitment and activation, LDL accumulation and oxidation, migration and proliferation of smooth muscle cells and fibrosis is the gradual growth and restructuring of the lesion which becomes a core of lipid and necrotic tissue overlaid by a fibrous cap – an advanced atherosclerotic plaque (Ross 1999).

1.2.2: Atherosclerosis – Histology

The term “atherosclerosis” derives from the Greek “athero” meaning wax and “sclerotic” meaning hard which is a very accurate description of an atherosclerotic plaque with its typically necrotic, lipid-rich core encased in a fibrous cap over the plaque’s luminal edge. It wasn’t until the mid-1990s that the terminology used to defined atheromatous plaques was defined by the American Heart Association (AHA) consensus group as shown in Table 2 (Stary, Chandler et al. 1995).

AHA type	Histological description
Early lesions	
Type I	Isolated groups of lipid-filled macrophages (foam cells). Normal macroscopical appearance.
Type II	Increased number of foam cells stratified into layers together with foamy smooth muscle cells. Fatty streaks are visible on gross examination.
Pre-atheroma/ Intermediate lesions	
Type III	Histological appearance intermediate between early fatty streaks and atheroma. Increased lipid content. Organized layers are observed. Erosion can occur.
Advanced atherosclerotic plaques	
Type IV- Atheroma	<u>Early necrosis</u> : focal macrophage infiltration into lipid-rich areas. Overlying fibrous cap. <u>Late necrosis</u> : loss of matrix and extensive cellular debris. Overlying fibrous cap.
Type Va- Fibroatheroma	Thin, fibrous cap (<65µm). Extensive macrophage and lymphocyte infiltrate with rare SMCs. Large underlying necrotic core. Intra-plaque haemorrhage/ fibrin may be present.
Type Vb: Calcified	Abundant mineralization. Decreased inflammatory infiltrate.
Type Vc- Fibrotic	Abundant collagen fibres. Minimal lipid accumulation.
Complicated atherosclerotic plaques	
Type VI	Presence of defects on the luminal surface (erosions, fissures, ruptures) leading to the formation of haematomas or thrombi.

Table 2: AHA Consensus of Atherosclerotic Lesions Based on Morphologic Descriptions (adapted from Stary et al.).

The two non-progressive lesions (Type I and II) usually appear during the first decades of life and have little impact on the arterial lumen diameter. The lipids found in these early lesions are mainly LDL which is thought to derive from circulation after being entrapped in the subendothelial space (Napoli, D'Armiento et al. 1997, Khalil, Wagner et al. 2004).

This first attempt at a formal, systematic description of the various clinically relevant atherosclerotic lesions has been repeatedly reviewed and adapted to accommodate specific types of plaques thought to be particularly relevant in certain types of CVD. One of the most relevant adaptations was proposed by Virmani *et al.* (Virmani, Kolodgie et al. 2000) who found to be lacking, in the original AHA classification, alternative entities which are mainly relevant for the occurrence of coronary thrombosis, namely the erosion and the calcified nodule. The recognition of particular types of plaques predominantly associated with the occurrence of acute coronary syndromes (ACS) led to the development of the concept of “stable vs. unstable plaque”. Atherosclerotic plaques which remain clinically quiescent are deemed “stable” as opposed to vulnerable or unstable plaques which are prone to erosion and have therefore a greater likelihood of leading to acute thrombus formation (Davies and Thomas 1985). It is now believed that the majority of ACS arise from the transformation of a stable plaque into an unstable one (Davies and Thomas 1985, Farb, Burke et al. 1996, Burke, Farb et al. 1997). Finn *et al.* (Finn, Nakano et al. 2010) recently published a review on the concept of vulnerable/ unstable plaque. The exposure of highly thrombogenic intra-plaque elements, such as tissue factor, to the blood flow leads to activation of the clotting cascade and ultimately to thrombus formation. To date, three histological phenotypes have been associated with luminal thrombosis: plaque rupture, plaque erosion and calcified nodule. Therefore, a plaque should be considered vulnerable when its morphology makes it prone to any of these events. The histological plaque phenotype thought to be associated with greater instability is the Thin-Cap Fibroatheroma (TCFA) or Type Va according to the AHA classification. These plaques typically have large necrotic cores with a rich-inflammatory infiltrate which is surrounded by a frail fibrous cap which usually <65 µm thick. Similar findings were obtained when assessing carotid plaques as demonstrated by Carr *et al.* who showed that there was a greater prevalence of plaque rupture, fibrous cap thinning and fibrous cap foam-cell infiltrates in symptomatic carotid stenosis compared to patients with asymptomatic stenosis (Carr, Farb et al. 1996).

The thinning of the fibrous cap is thought to arise from a decrease in smooth muscle cells and type I and II collagen fibres induced by the action of proteolytic enzymes produced by activated macrophages within the necrotic core (Moreno, Falk et al. 1994, Libby 1995, Shah, Falk et al. 1995). Bassiony *et al.* showed in a series of 99 endarterectomy plaques the

number of macrophages infiltrating the fibrous cap was three-times greater in symptomatic common carotid plaques compared to asymptomatic and this was not influenced by the size of the necrotic core area (Bassiouny, Sakaguchi et al. 1997).

Other factors related to plaque morphology can also play a role. It was found that a closer proximity of the necrotic core to the lumen was two-times higher in symptomatic carotid plaques compared to asymptomatic plaques (Bassiouny, Sakaguchi et al. 1997). Moreover, the presence of neovessels within the plaque itself or its fibrous cap was significantly associated with symptomatic carotid plaques. Furthermore, the presence of neo-vascularization was found to be associated with increased plaque necrosis and rupture (McCarthy, Loftus et al. 1999). Anatomical location of plaque or the shear-stress forces produced by blood flow may also contribute for the development of vulnerable plaques (Gijssen, Wentzel et al. 2008, Finn, Nakano et al. 2010).

Unstable plaques are more frequently found in the proximal coronary arteries and are more common in patients with high total cholesterol (t-cholesterol)/ high density lipoproteins (HDL) ratio, in women >50 years and in patients with higher levels of high-sensitivity C-reactive protein (CRP) (Virmani, Burke et al. 2006).

Ultimately, CVD is clinical expression of the presence of atherosclerotic plaques, ranging from an asymptomatic state to chronic disease (stable angina and chronic peripheral ischaemia) to potentially life-changing acute events (myocardial infarction - MI, stroke). It is the underlying histology of plaques, along with their preferential location, which is responsible for their broad clinical presentations. Thus, these depend mainly on the progression of atherosclerosis in the vessel wall itself i.e., a progressive reduction of arterial flow due to a slowly growing plaque leads to chronic symptoms, which tend to gradually worsening symptoms such as intermittent claudication whereas plaque rupture and thrombus formation can quickly lead to major events like ACS (Virmani, Burke et al. 2006, Finn, Nakano et al. 2010).

1.2.3: Traditional risk factors and novel biomarkers

One of the greatest challenges for both researchers and clinicians with a combined interest in CVD and SLE is to identify at-risk patients before the onset of clinically evident atherosclerosis. In order to achieve this, it is important to establish a risk profile, which in turn frequently means identifying risk factors. A risk factor can be defined as a measurable biological characteristic of an individual that precedes a well-defined outcome, predicts that

outcome and is in the direct biological causal path (Balagopal, de Ferranti et al. 2011). The term risk factor is frequently mis-used to describe a biomarker which is in fact a biological indicator for processes that are involved in developing the disease that may or may not be causal (Balagopal, de Ferranti et al. 2011). In other words a biomarker can only be considered a risk factor when there is evidence to support the idea that an intervention results in a change of risk (Stampfer, Ridker et al. 2004). The major risk factors associated with the development of CVD have all been described many years ago and their relevance and impact remain unparalleled (Dawber, Kannel et al. 1959, Kannel, Dawber et al. 1961). Variables such as older age, male gender, hypertension, diabetes and hypercholesterolemia have thus long been viewed as the cornerstone of any CVD-risk assessment algorithm. However, as previously stated the traditional risk factors grossly underestimate the CVD risk in the context of auto-immune diseases, in particular SLE. Although this is a well-established fact, the traditional risk factors are beginning to be viewed in a different light. For example, the concept of vascular age as opposed to chronological age is starting to emerge. Based on the observation about the large variability in the incidence of CVD among individuals of the same age, several population studies based either on coronary artery calcium score (CACS) (Schisterman and Whitcomb 2004, Nasir, Vasamreddy et al. 2006) or carotid-intima-media thickness (CIMT) (Stein, Fraizer et al. 2004, McClelland, Nasir et al. 2009) have supported the notion that vascular age may be a better risk predictor than chronological age (Budoff, Nasir et al. 2009). Similarly, the way hypercholesterolemia is viewed is also changing, in particular elevated circulating LDL. In simple terms, the way to assess LDL-related risk is based on the Friedwald method which assesses total cholesterol and TG enzymatically and HDL after precipitation of apolipoprotein B-containing lipoproteins. LDL cholesterol is then calculated as the cholesterol fraction remaining after HDL and VLDL-cholesterol are subtracted from the total cholesterol. Besides its well-known limitations, namely the presence of very high TG levels ($>4.5\text{mmol/L}$), the measurement of LDL-cholesterol concentration represents a limited surrogate marker of LDL-related atherogenicity. LDL particles and not the cholesterol contained in their hydrophobic core interact with the vessel wall during plaque formation and progression (Barter, Ballantyne et al. 2006). For any given LDL-cholesterol level, the LDL particle number may vary depending on the quantity of cholesterol carried by each particle. Cromwell et al showed in a study of the Framingham cohort that discordance between LDL-cholesterol and LDL-particle number is found in about a fifth of individuals and that when this happens, LDL-particle was strongly associated with outcomes while LDL-cholesterol had no relation (Cromwell, Otvos et al. 2007). These two examples of new ways to look at the traditional risk factors may improve their predictive value when assessing patients with SLE.

Over the last decade, the search for alternative, more sensitive and specific CVD risk factors has been a major focus of interest. Several biomarkers from serological variables such as CRP, plasma fibrinolysis activation markers and vitamin D to genetic profiling and pulse wave velocity indices (brachial-ankle and/or carotid femoral) have cyclically been proposed as candidate risk factors. However, none of these new biomarkers has been able to pass the high hurdle of causality and have therefore had limited success in expanding the epidemiological profile (Fruchart, Nierman et al. 2004, deGoma, Knowles et al. 2012).

Ultimately, the real atherosclerotic burden can best be established by using imaging methods which allow for a comprehensive vascular assessment through plaque measurement and characterization.

1.2.4: Cardiovascular disease risk stratification

Regardless of the vascular territory predominantly affected by atherosclerosis, identifying asymptomatic patients with already significant subclinical disease is the key for primary prevention. It was based on this premise that risk stratification algorithms have repeatedly been developed and improved in a bid to calculate the future risk of cardiovascular events with the highest predictive value possible. The main risk stratification tools are based on levels of well-established CVD risk factors such as age, sex, serum cholesterol, smoking and blood pressure. The Framingham risk score (Anderson, Odell et al. 1991, Wilson, D'Agostino et al. 1998) is the most widely used, but others have also been developed, namely the Reynolds score (Ridker, Paynter et al. 2008) which includes high-sensitivity CRP, the Prospective Cardiovascular Munster (PROCAM)(Assmann, Cullen et al. 2002) and the Sheffield table system (Haq, Jackson et al. 1995). These different methods are similar in their overall low sensitivity and specificity as they exclude various emerging, genetic and otherwise unknown risk factors. Moreover, the vast majority of these indexes fail to take into account factors which have been shown to influence CVD risk such as SLE or RA. An exception for this statement is the QRISK algorithm which takes into account the diagnosis of RA as one of its risk factors for life-long cardiovascular disease (Hippisley-Cox, Coupland et al. 2008). To my knowledge there are no other risk assessment tools which account either for RA or other types of systemic inflammatory conditions, specifically SLE. When applied to patients with chronic inflammatory conditions such as SLE, these risk stratification tools can be useful in identifying high risk individuals (risk of CVD-related events >20% over 10 years). However, they fail to detect the majority of those patients with SLE who eventually develop CVD because they are labelled as low risk by the same set of criteria. This discrepancy is due to a combination of flaws in the different scoring systems. In the context of SLE, the best

example is the Framingham risk score: as it is heavily weighted for age and gender, the risk of coronary artery disease in young women with SLE is greatly underestimated.

Optimizing early medical treatment and controlling modifiable risk factors has been shown to reduce cardiovascular events in both symptomatic and asymptomatic patients (Wright and Flapan 1994, Downs, Clearfield et al. 1998). Patients with subclinical atherosclerotic disease would ideally be identified before any events occur, thus enabling primary prevention strategies and minimizing mortality and morbidity.

As will be discussed in detail in section 1.5, carotid US has been shown to be a sensitive, non-invasive, reproducible imaging study to detect atherosclerosis, predict its clinical complications and monitor its progression and regression in response to treatment. IMT has been shown to correlate with several traditional and emerging CVD risk factors (Ebrahim, Papacosta et al. 1999, O'Leary and Polak 2002) and although its risk prediction value is debatable, its use in assessing asymptomatic patients for CVD has been suggested by the American Heart Association and the European Society of Hypertension- European Society of Cardiology (Greenland, Abrams et al. 2000, European Society of Hypertension-European Society of Cardiology Guidelines 2003). In addition to IMT measurement, another, an arguably even more useful indicator of CVD risk is the presence of plaque as will be discussed later (Chapter 1.5.2: Importance of Vascular US in CVD Assessment).

1.2.5: Cardiovascular Disease and SLE

Besides the potentially multisystem involvement directly related with its autoimmune nature, SLE is also one of the strongest known risk factors for CVD (D'Cruz, Khamashta et al. 2007, Pennell and Keenan 2011). The range of cardiovascular involvement in SLE is broad including atherosclerosis, vascular inflammation/vasculitis, Raynaud's phenomenon, endothelial dysfunction and a procoagulant tendency associated with the presence of antiphospholipid antibodies. Focusing on atherosclerosis-related cardiovascular disease, Magder et al reported a 2.66-fold increased risk of CVD in the Hopkins Lupus Cohort compared with the Framingham controls (Magder and Petri 2012). The impact of CVD-related events on both mortality and morbidity is tremendous: the incidence of coronary artery disease both in its acute (MI) and chronic (angina, chronic heart failure) forms is over 7 times greater in patients with SLE than in healthy controls, even after accounting for traditional CVD risk factors, including sex, age and lipid profile (Manzi, Meilahn et al. 1997, Esdaile, Abrahamowicz et al. 2001). Impressively, younger female patients with SLE (age 35-44 years) have an over 50 times greater risk of having a MI compared with the

Framingham dataset (Manzi, Meilahn et al. 1997). When looking at the typical bimodal mortality pattern found in SLE, the real importance of CVD becomes apparent: a decade after the diagnosis of SLE, one of the leading causes of death is MI (Urowitz, Bookman et al. 1976, Abu-Shakra, Urowitz et al. 1995, Urowitz, Gladman et al. 1997, Bernatsky, Boivin et al. 2006). Patients with SLE also have a greater risk for stroke, with an overall prevalence that can reach 20%, as well as high recurrence rate and greater mortality than matched controls (Kitagawa, Gotoh et al. 1990).

The reason why SLE is such a dramatic risk factor for CVD is yet to be fully explained. It appears that in patients with SLE, there is an accelerated atherosclerotic process (Asanuma, Oeser et al. 2003, Roman, Shanker et al. 2003) which seems to arise from a complex interplay of traditional and lupus-specific risk factors (Esdaile, Abrahamowicz et al. 2001, Bessant, Hingorani et al. 2004, Selzer, Sutton-Tyrrell et al. 2004, Bessant, Duncan et al. 2006). On the one hand, the presence of longstanding systemic inflammation associated with persistently active SLE could contribute to plaque formation and disruption. On the other hand, it has been found that patients with lupus have a high prevalence of traditional CVD risk factors such as hypertension, altered lipid profile and impaired glucose tolerance (MacGregor, Dhillon et al. 1992, Esdaile, Abrahamowicz et al. 2001, Bessant, Duncan et al. 2006), which to some extent may be the result of chronic treatment with corticosteroids (Petri, Perez-Gutthann et al. 1992, Karp, Abrahamowicz et al. 2008). However, to date no undisputed correlation has been found between corticosteroid use and atherosclerosis in SLE. Magder et al reported that the risk of cardiovascular events is increased by two to 5-fold for prednisolone doses above 10mg and 20mg respectively (Magder and Petri 2012). This could be due to the effects of corticosteroids on BP and lipid profile, as reported for the Hopkins Lupus Cohort where a daily dose of 10mg of prednisolone was associated with an average total cholesterol rise of 0.19mmol/L, a weight gain of 5.5lbs and an increased BP by 1.1mmHg (Petri 2000). However, there are also conflicting data suggesting that there is an increased CVD risk among patients who are under treated with steroids, which could imply that poorer disease control is more relevant in determining a higher CVD risk than steroid treatment *per se* (Asanuma, Oeser et al. 2003). In summary, the raised CVD risk is likely to be due to interplay between direct vascular damage arising from inflammatory phenomena associated with SLE, such as vasculitis and immune-complex mediated endothelial cell damage; treatment-related factors such as steroid-induced hyperlipidaemia, hypertension and obesity and other SLE-independent factors, such as homocysteine levels, smoking and family history. Contributing factors indirectly related with SLE, namely nephrotic syndrome secondary to lupus nephritis and the presence of anti-phospholipid antibodies should also be considered (Petri, Perez-Gutthann et al. 1992, Petri, Roubenoff et al. 1996). Although the

majority of SLE-study groups report an association between CVD-related morbidity and mortality and anti-phospholipid antibody positivity (Toloza, Uribe et al. 2004, Gustafsson, Simard et al. 2012, Magder and Petri 2012), there are fewer studies where this association was not found to be significant (Roman, Shanker et al. 2003).

Treatment can also affect CVD-risk in a positive way: the use of hydroxychloroquine (HQ) for example, may play a potentially protective role as it has been shown to be associated with lower cholesterol levels, independently of other variables (Petri, Lakatta et al. 1994, Cairolì, Rebella et al. 2012) and may potentially counteract the negative effects of prednisolone on lipid profile (Petri, Lakatta et al. 1994). Furthermore, HQ appears to inhibit platelet aggregation and decrease the binding of anti- β 2GP1 to phospholipid layers thus decreasing the risk of thrombosis (Espinola, Pierangeli et al. 2002, Rand, Wu et al. 2008, Rand, Wu et al. 2010).

One of the main issues when addressing CVD in the context of SLE is the poor performance of standard stratification tools based on the Framingham risk equation (Bessant, Hingorani et al. 2004, O'Neill, Pego-Reigosa et al. 2009) as previously discussed. The inability to assess accurately the actual CVD risk for each individual patient has led to an inability to shift CVD-associated mortality and morbidity in SLE, opposing the trend observed for other causes of mortality such as lupus nephritis (Bjornadal, Yin et al. 2004, Bernatsky, Boivin et al. 2006). There is a need for alternative ways to assess and identify patients with SLE with potentially higher risk for developing CVD, with particular emphasis in non-invasive screening tools aimed at detecting subclinical atherosclerosis. Ahmad et al. published a study focusing on assessing the strength of the association between traditional cardiovascular risk factors and presence of carotid plaque assessed by B-mode ultrasound (Ahmad, Shelmerdine et al. 2007). The US findings of 200 women with SLE were compared with those of 100 healthy controls. An increased prevalence of plaque was observed among the SLE cohort, particularly on the internal carotid artery (11% vs 4%; $P < 0.05$). Traditional risk factor models performed less well in SLE compared with controls but when using a multivariable model which exclusively included SLE factors (including azathioprine use and APL), a significant performance improvement was noted. The authors thus conclude that it is the Lupus phenotype that more sensitively identifies patients with greater risk for atherosclerosis suggesting that SLE-specific factors are likely to have an additive effect over traditional cardiovascular risk factors rather than sensitizing patients.

The use of SLE-specific stratification algorithms has been proposed, with particular emphasis on composite risk-assessment scores based on traditional risk factors and novel biomarkers. A good example is the PREDICTS score proposed by McMahon et al who argue that this is a useful tool to improve the identification of patients with SLE who have a greater

risk for CVD (McMahon, Skaggs et al. 2014). This study included 210 patients with SLE and 100 age-matched healthy control subjects who underwent 2D carotid US to assess the presence of atherosclerotic plaque and the IMT at two time points (0 and 2 years). For all the participants, complete data on traditional CVD risk factors (age, sex, diabetes hypertension, lipid profile) and treatment where applicable was collected (including cumulative steroid use). Moreover, all the participants were tested for a panel of serological variables that included pro-inflammatory HDL (piHDL), leptin, serum TNF-like weak inducer of apoptosis (sTWEAK), serum homocysteine, high-sensitivity CRP (hsCRP), adiponectin and apolipoprotein A1. Although some variables were shown to correlate significantly with the presence of plaque, namely the presence of diabetes, age equal or greater than 48 years, high piHDL, high leptin and high sTWEAK levels, no single variable demonstrated an ideal combination of sensitivity and specificity. Consequently, a high risk PREDICTS profile was proposed based on the presence of at least 3 positive biomarkers or a combination of diabetes plus at least one of the biomarkers considered. Based on this definition, patients who had the high risk profile had a 28 fold increased odds for the presence of plaque and increased IMT progression, with a 79% specificity and a 89% sensitivity (negative predictive value 89% and positive predictive value 64%)(McMahon, Skaggs et al. 2014). In summary, the widely accepted notion is that in SLE the combination of disease-specific and traditional CVD risk factors lead to an accelerated atherosclerotic process, which in turn contributes to plaque destabilization ultimately leading to plaque disruption and the occurrence of potentially fatal acute events.

1.3: Lipoprotein metabolism

1.3.1: Overview of lipoprotein metabolism

Lipoproteins (Lp) are large macromolecular complexes that transport hydrophobic lipids through bodily fluids and tissues, playing an essential role in the absorption of dietary cholesterol and long chain fatty acids, as well as in the transport of triglycerides (TG) and cholesterol to and from the liver and peripheral tissues (Longo DL (editor) 2011). Lp contain a core of hydrophobic lipids (TG and cholesteryl esters) surrounded by hydrophilic lipids (phospholipids and unesterified cholesterol) and proteins.

There are 5 major groups of Lp which differ mainly in their protein density i.e. the protein to cholesterol ratio. Chylomicrons are the lowest density lipoproteins followed by very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), low density lipoproteins (LDL) and high density lipoproteins (HDL). HDL is the smallest and most dense Lp whereas chylomicrons and VLDL are the largest and least dense. Most plasma TG are transported in chylomicrons or VLDL while cholesterol is transported as cholesteryl esters in LDL or HDL.

The proteins associated with Lp are apolipoproteins (Apo) which are required for the assembly, structure and function of Lp. ApoA1 is synthesized in the liver and intestine and it is found on virtually all HDL particles while ApoB is the major structural component of chylomicrons, VLDL, IDL and LDL. ApoE is present in chylomicrons, IDL and LDL and plays a critical role on the metabolism and clearance of TG-rich particles. There are two main Lp metabolic pathways: the endogenous and the exogenous pathway.

The exogenous pathway is responsible for the efficient transport of dietary lipids through which dietary cholesterol and fatty acids (FA) are esterified in the small intestine by the enterocytes to form cholesteryl esters, while longer chain FA are incorporated firstly in TG and secondly into chylomicrons. Chylomicrons are extensively processed in the peripheral tissues through the action of lipoprotein lipase (LpL) releasing free FA which are in turn either oxidized to generate energy or re-esterified and stored as TG (Longo DL (editor) 2011). As the hydrophobic core is hydrolysed and the hydrophilic lipids (cholesterol and phospholipids) and surface Apo are transferred to HDL, chylomicrons gradually shrink in size (chylomicron remnants) and are finally removed from the circulation at the liver through an ApoE mediated process.

The endogenous pathway describes the hepatic secretion of ApoB-containing Lp and their metabolism. As with chylomicrons, the TG contained in VLDL are hydrolysed by LpL on peripheral tissues (muscle and adipocytes) gradually becoming IDL. The cholesterol content of IDL is almost all removed by the hepatocytes by LDL-receptor (LDL-R)-mediated endocytosis via binding to ApoE and the remainder of IDL is remodelled to form LDL. During this process, most of the TG are hydrolysed and all Apo (except ApoB-100) are transferred to other Lp. Finally, the clearance of LDL is done mainly on the liver through LDL-R mediated endocytosis.

Figure 1 illustrates the overall Lp metabolism, depicting both the exogenous and endogenous pathway.

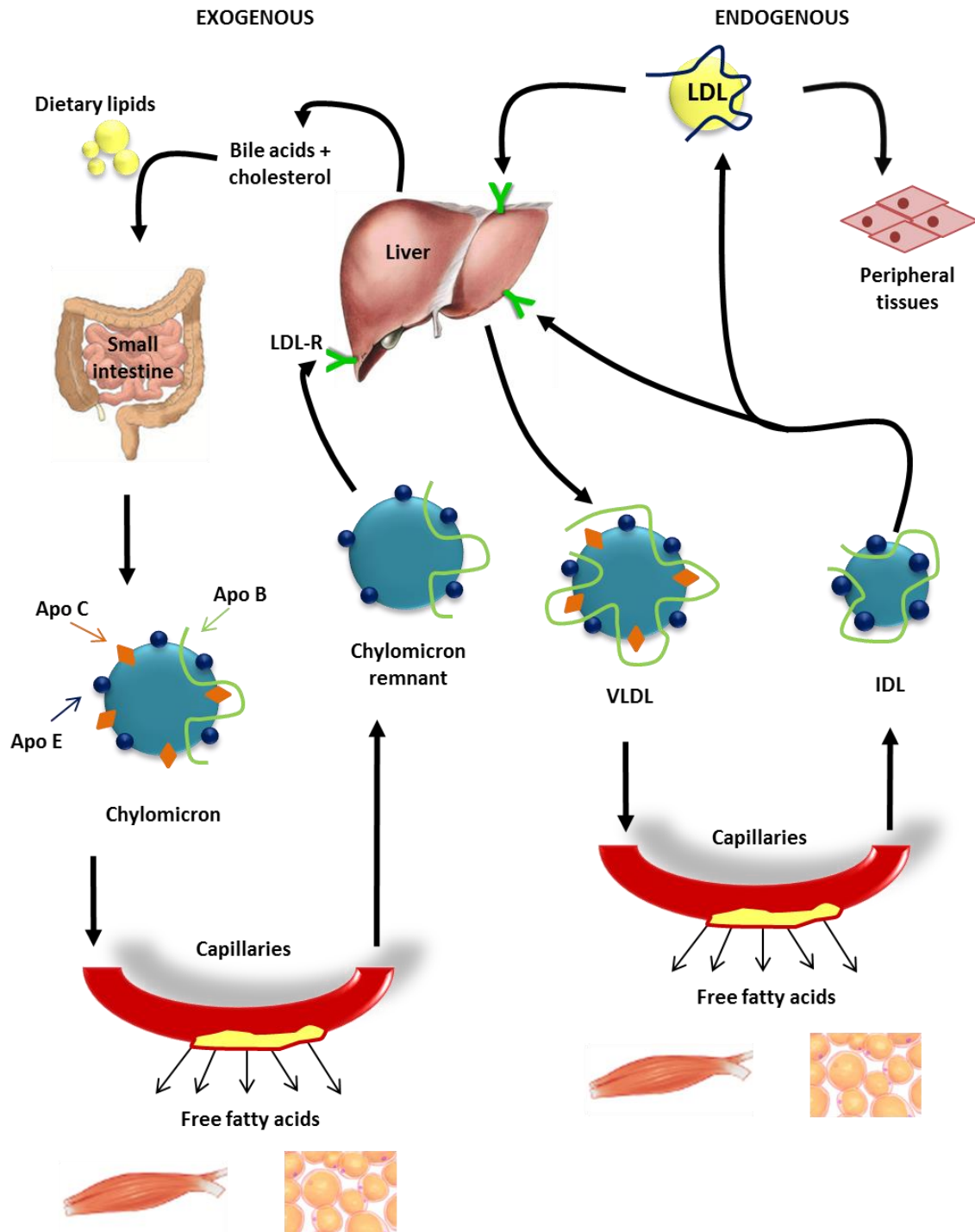


Figure 1: The exogenous and endogenous lipoprotein metabolic pathway: the exogenous pathway transports dietary lipids to the periphery and the liver. The endogenous pathway transports hepatic lipids to the periphery (LPL: lipoprotein lipase; LDL: low density lipoprotein; IDL: intermediate density lipoprotein; VLDL: very low density lipoprotein; LDL-R: LDL-receptor).

1.3.2: Structure and physiological role of HDL

Although all nucleated cells synthesise cholesterol, only hepatocytes and enterocytes can effectively excrete cholesterol from the body into bile or the intestinal lumen. In peripheral cells, cholesterol is transported from the plasma membranes to the liver by a process termed “reverse cholesterol transport”. This multi-step process results in the net movement of cholesterol from peripheral tissues back to the liver via the plasma compartment and relies to a great extent on HDL function.

Nascent HDL are synthesized in the liver or intestine as complex of protein and phospholipids and their most abundant component is a 243 amino acid chain called apolipoprotein A1 (ApoA1). Initially, HDL is a flattened, cholesterol free lipoprotein particle which gradually incorporates cholesterol into its structure via trans-membrane efflux mediated by ATP-binding cassette protein A1 (ABCA1). The ability to achieve cholesterol uptake depends on the action of a plasma enzyme, lecithin-cholesterol acetyltransferase (LCAT) which converts free cholesterol into hydrophobic cholesteryl esters which are then sequestered into the lipoprotein core finally composing the spherical HDL (Devlin 2011) as represented in Figure 2.

Circulating HDL gradually increases its size and ultimately delivers its cholesterol content to the liver and steroid-producing organs (adrenals, ovaries and testis). The removal of cholesterol from within the HDL structure is mediated by the cholesteryl ester transfer protein (CETP) which exchanges the cholesteryl esters carried by HDL for the triglycerides carried by ApoB-containing Lp that are further processed into LDL finally reaching the LDL-R pathway. Alternatively, HDL cholesterol can also be directly up taken by the hepatocytes via the scavenger receptor class BI (SR-BI). The remodelling of HDL also occurs in the plasma compartment as phospholipids are transferred from other circulating Lp to HDL. Following CETP-mediated lipid exchange, HDL is maximally enriched with TG thus becoming an optimum substrate for either hepatic or endothelial lipase which hydrolyse TG and phospholipids yielding smaller, flattened HDL particles which are then released back into circulation to resume this cycle (Devlin 2011).

Figure 3 depicts the overall HDL metabolism and reverse cholesterol transport.

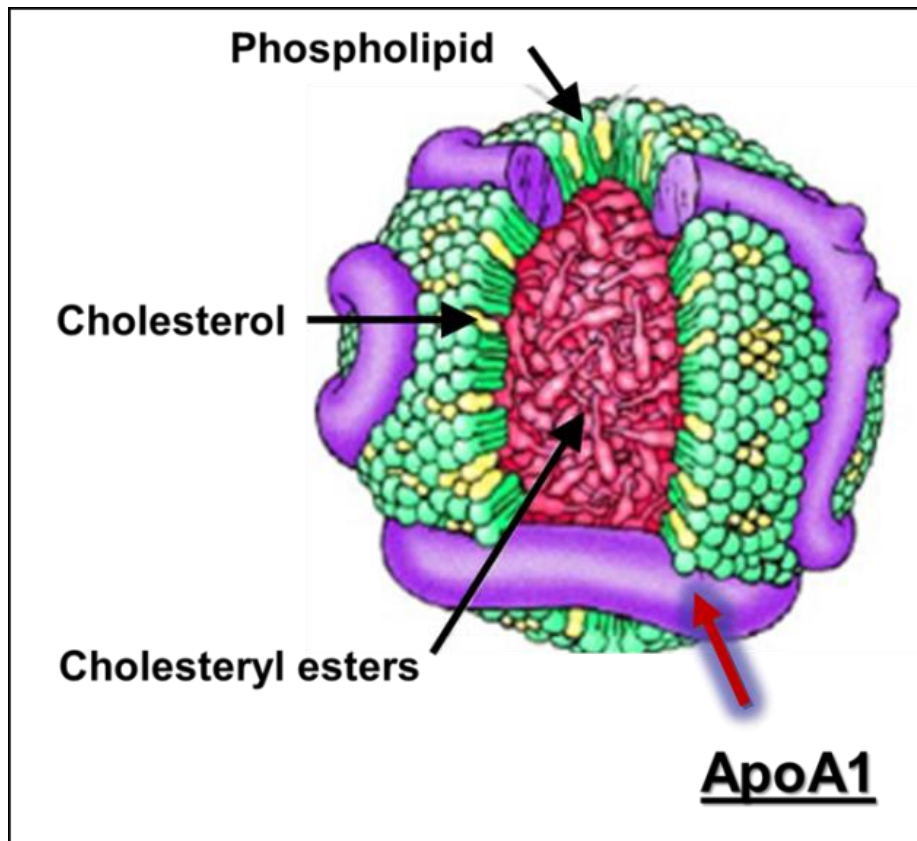


Figure 2: Schematic representation of the structure of HDL: the main structure is formed by apolipoprotein A1 and to a lesser extent by phospholipids. Cholesterol is transported mainly as cholesteryl esters located inside the HDL particle core.

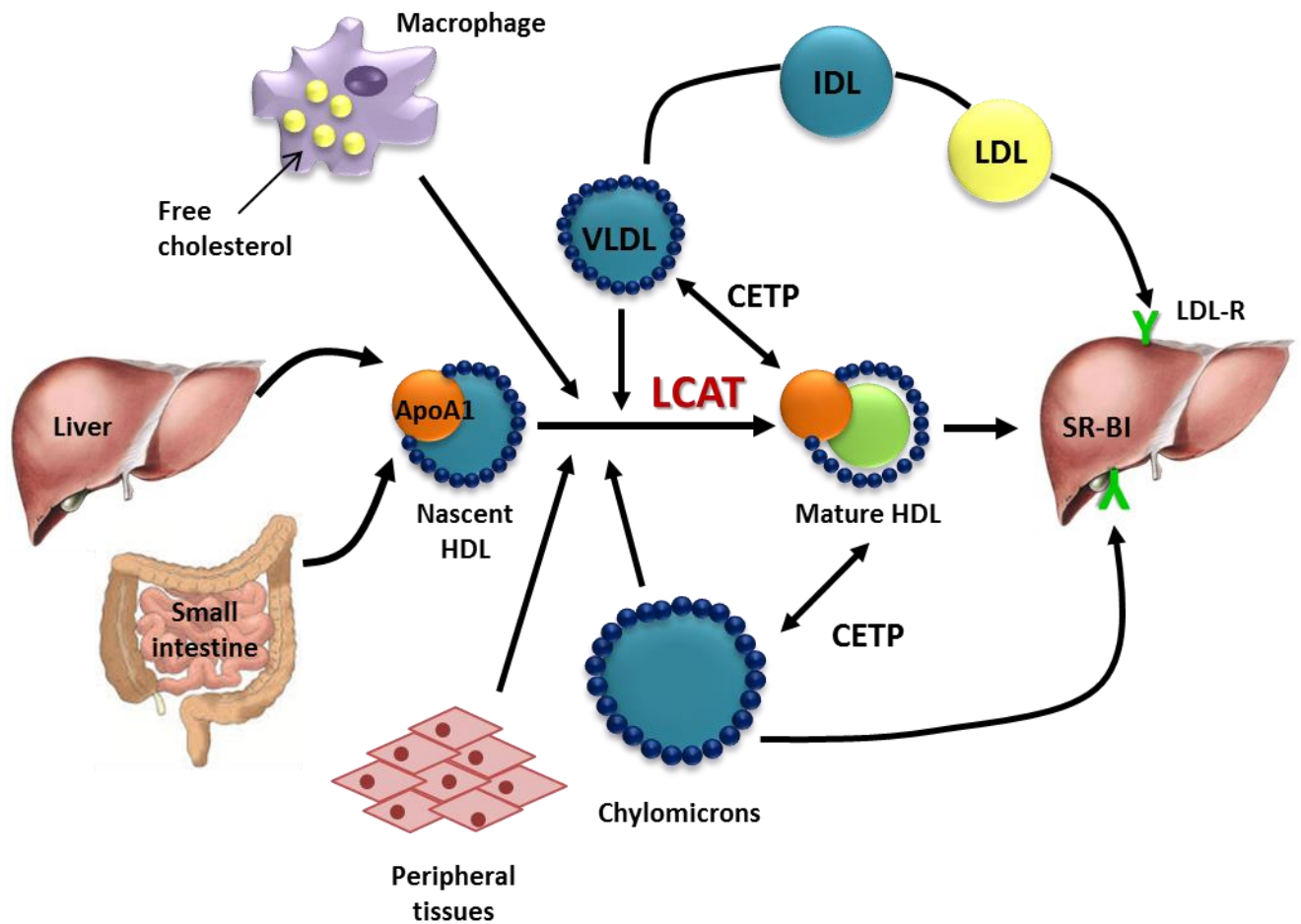


Figure 3: HDL metabolism and reverse cholesterol transport: the liver and the intestine produce nascent HDL particles. Free cholesterol is acquired from macrophages and other peripheral tissues and esterified by LCAT thus forming mature HDL particles. HDL cholesterol can then be taken up by the liver via the SR-BI or alternatively, cholesteryl esters can be transferred from HDL to VLDL and chylomicrons by CETP which in turn are taken up by the liver (HDL: high density lipoprotein; LDL: low density lipoprotein; IDL: intermediate density lipoprotein; VLDL: very low density lipoprotein; CETP: cholesteryl ester transfer protein; LCAT: lecithin-cholesterol acyltransferase, LDL-R: LDL-receptor).

HDL has long been viewed as having a protective role in atherosclerosis, mostly due to its central role in reverse cholesterol transport, thus contributing to the mobilisation of cholesterol from foam cells present in atherosclerotic plaque, leading to its stabilization and even regression. However, HDL has other beneficial actions which have gradually been recognized, namely anti-thrombotic, anti-oxidant and anti-inflammatory properties (Ames, Batuca et al. 2010, Di Bartolo, Nicholls et al. 2011). *In vitro* models show that HDL inhibits endothelial expression of proinflammatory adhesion molecules such as VCAM and chemokines, reducing monocyte recruitment. HDL anti-oxidant role is mainly mediated by paraoxonase 1 (PON1) which prevents the oxidation of LDL present in the subendothelial space (Batuca, Ames et al. 2007, Hahn 2010). The paraoxonase family comprises 3 isoenzymes: PON1, PON 2 and PON 3. PON 1 is synthesized in the liver and transported along with circulating HDL, although it is also present in the arterial wall. Its main function is to prevent LDL oxidation and PON1 deficient mice are highly susceptible to developing atherosclerosis. PON-1 is stabilized by apoA1 and its levels decrease with age and are increased with treatment with lipid-lowering agent such as statins (Senti, Tomas et al. 2001, Batuca, Ames et al. 2007). PON2 is a constitutively expressed intracellular protein which protects the cells against oxidative stress. PON3 function and activity are less well known, but it appears to mirror PON1, as it circulates attached to HDL once it is synthesized in the liver. Moreover, its substrate isn't strictly LDL and, as opposed to PON1, its levels are not regulated by the presence of oxidized lipids.

Considering that the presence of oxidized LDL is one of the main stimuli for endothelial cell activation and monocyte recruitment, this action confers to PON1 an exceptional role in protecting the integrity of the arterial endothelium. However, for this PON1-mediated HDL effect to occur, apoA1 structure must be intact as it is responsible for transporting PON1 in its functional conformation (Mackness, Hunt et al. 1997).

As its main structural component, apoA1 is pivotal to HDL function and therefore its integrity is essential to normal lipid homeostasis (Hahn 2010, O'Neill, Giles et al. 2010, Vuilleumier, Bratt et al. 2010).

The protective role of apoA1 has been underlined by recent studies. In animal models, the infusion of high-dose ApoA1, or its over-expression, leads to reduction of inflammation (i.e. macrophage content) in experimental atheroma. Moreover, it also appears to improve endothelial dysfunction, decrease oxidized LDL and reduce monocyte recruitment, regardless of the effect on HDL levels. Recent studies suggest that the use of apoA1

mimetic peptides may offer the same benefits and could potentially be considered as a treatment option in the future (Di Bartolo, Nicholls et al. 2011).

1.3.3: Anti-HDL antibodies, anti-ApoA1 antibodies and dysfunctional HDL

As discussed before, although it is clear that there is an increased CVD risk in SLE, no straightforward culprit has been identified. As previously discussed, the impact of SLE-associated factors has to be considered in conjunction with traditional risk factors as no unequivocal link between various novel biomarkers and the atherosclerotic burden has been established (Lerner and Kannel 1986, Roman, Shanker et al. 2003).

In the general population, high levels of t-cholesterol, LDL and TG as well as low levels of HDL are independently associated with increased cardiovascular risk, particularly among women (Pearson, Blair et al. 2002). Several studies have shown that patients with SLE have a more atherogenic lipid profile, namely with reduced HDL and raised TG levels compared with control subjects (Ettinger, Goldberg et al. 1987, Borba, Carvalho et al. 2006). Furthermore, oxidative modifications of LDL including β_2 -glycoprotein 1 oxidized LDL complexes which have been identified in patients with SLE are associated with arterial thrombosis (Kobayashi, Kishi et al. 2003). Moreover, altered lipid profile has also been shown to influence non-CVD related outcomes, particularly regarding adverse renal outcomes (Tisseverasinghe, Lim et al. 2006).

The pathogenesis of the altered lipid profile observed in SLE is not yet fully established, but it is thought to be multifactorial. The presence of sustained inflammation most likely plays a central role, namely through the increase in inflammatory cytokines such as TNF α and IL-6 which have been shown to influence lipid levels, shifting them towards an atherogenic profile (Svenungsson, Gunnarsson et al. 2003, Chung, Avalos et al. 2007).

One of the proposed mechanisms, which may contribute to the presence of accelerated atherosclerosis in SLE, is the presence of certain auto-antibodies which target components of the lipid metabolism cycle. For the purposes of my thesis, I will focus on two main groups: anti-ApoA1 and anti-HDL antibodies.

ApoA1 is the main component of HDL whose protective role relies greatly on its presence and structural integrity. Furthermore, apoA1 is essential to stabilize PON-1 allowing it to play its role in preventing lipid peroxidation and oxidised LDL formation (Vuilleumier, Charbonney et al. 2008). The presence of anti-apoA1 could have a deleterious impact at several levels: 1) impairment of normal HDL function contributing to the presence of dysfunctional or inflammatory HDL; 2) impaired anti-oxidant capacity by compromising PON1 activity; 3)

formation and deposition of immunocomplexes leading to tissue damage. However, alternative mechanisms through which anti-ApoA1 may contribute to enhanced atherogenesis have also been proposed.

Delgado Alves et al reported on the effects of anti-apoA1 and anti-HDL on PON-1 function in patients with SLE and primary APS. They found that on the SLE subgroup PON-1 activity was reduced; an inverse correlation between PON-1 activity and anti-HDL levels also emerged. Furthermore, PON-1 levels paralleled serum total antioxidant capacity, while anti-HDL was negatively correlated with the latter (Delgado Alves, Ames et al. 2002). These findings were supported by further work which confirmed the inverse correlation between the presence of anti-HDL and reduced PON-1 activity. A similar correlation was noted between anti-apoA1 and reduced PON-1 activity (Batuca, Ames et al. 2007, Batuca, Ames et al. 2009).

The atheroprotective role of ApoA1 is well established and there is evidence to support the idea that presence of anti-ApoA1 may contribute to the development of atherosclerosis in non-SLE patients, namely in patients with ACS and diabetes (Vuilleumier, Charbonney et al. 2008, Pagano, Satta et al. 2012, Teixeira, Cutler et al. 2012). Furthermore, Montecucco et al have shown that the presence of anti-ApoA1 IgG is associated with increased intra-plaque macrophage neutrophil and metalloproteinase content and inversely with collagen *in vivo*. This profile has been shown to be associated with increased plaque vulnerability and increased risk of plaque rupture, suggesting that the presence of anti-ApoA1 IgG may be associated with high-risk plaques (Montecucco, Vuilleumier et al. 2011).

Pagano et al showed that, in patients with MI, the presence of anti-apoA1 was associated with higher levels of IL 6, TNF α and metalloproteinase 9 (Pagano, Satta et al. 2012). *In vitro* studies suggested that this effect was mediated by stimulation of the TLR2/ CD14 pathway and hypothesised that this could be due to molecular mimicry, as there is a strong structural homology between apoA1 and TLR2 (Pagano, Satta et al. 2012).

The presence of anti-ApoA1 and anti-HDL in the sera of patients with SLE has been reported by different research groups (Dinu, Merrill et al. 1998, Delgado Alves, Ames et al. 2002, Alves and Ames 2003, Delgado Alves, Kumar et al. 2003, Batuca, Ames et al. 2007, Batuca, Ames et al. 2009, O'Neill, Giles et al. 2010).

Lahita et al noted that the levels of apoA1 were reduced in a cohort of patients with SLE compared with HC (Lahita, Rivkin et al. 1993). Follow-up work by Dinu et al showed that in 520 samples from 175 patients with either SLE or primary APS, antibodies directed against purified apoA1 could be found in 32.5% and 22.9% of samples respectively and the high

affinity of these antibodies for mature HDL molecules was confirmed. An association between anti-apoA1 and antiphospholipid antibodies (aPL), namely anti- β 2 GP1 was noted (Dinu, Merrill et al. 1998).

A strong correlation between titres of anti-apoA1 and anti-HDL antibodies has been noted (Delgado Alves, Kumar et al. 2003, Batuca, Ames et al. 2009, O'Neill, Giles et al. 2010), suggesting that apoA1 may be one of the key antigens for anti-HDL. However, this does not exclude the possibility of other potential targets within the HDL complex. In fact, the presence of anti-apoA1 is not necessarily accompanied by anti-HDL and vice versa. O'Neill et al reported that although there was a statistically significant correlation between anti-HDL and anti-apoA1 antibodies ($r= 0.387$), only 67% of samples positive for anti-apoA1 were also positive for anti-HDL (O'Neill, Giles et al. 2010).

Batuca et al showed that the presence of anti-apoA1 and anti-HDL correlated with impaired PON-1 activity, increased nitric oxide metabolites (modified Griess reaction) and reduced total anti-oxidant capacity. Furthermore, a positive correlation between these auto-antibodies and VCAM-1 and ICAM-1 was also noted. Finally, anti-ApoA1, anti-HDL and reduced PON-1 activity were associated with increased BILAG score and SLICC damage index (Batuca, Ames et al. 2009).

A link between disease activity and anti-HDL and anti-apoA1 antibodies was identified. O'Neill et al reported that anti-apoA1 and anti-HDL levels varied over time and that patients with persistently high disease activity had significantly higher levels than those with quiescent disease (O'Neill, Giles et al. 2010). The association between increased anti-apoA1 and anti-HDL IgG and reduced PON-1 activity with both clinically defined active disease and damage scores was supported by further studies (Batuca, Ames et al. 2009). No associations with treatment were reported by either group.

Another proposed mechanism which could explain the increased CVD risk in SLE is the presence of dysfunctional HDL (d-HDL). As previously discussed, HDL levels correlate inversely with the risk of atherosclerosis-related events (Navab, Anantharamaiah et al. 2006). However, when reviewing the data from the Framingham cohort, over 40% of events occurred in subjects with normal HDL levels (Gordon, Castelli et al. 1977) and subsequent studies have shown that a "normal" lipid profile does not necessarily exclude the risk of CVD as a significant number of events occur in subjects with normal HDL and LDL (Ansell, Navab et al. 2003, Navab, Ananthramaiah et al. 2005). Stemming from this observation, the hypothesis that there could be a dysfunctional form of HDL with compromised anti-inflammatory action and potentially a proatherogenic role emerged. The measurement of the qualitative function of HDL is based on its ability to prevent LDL oxidation and therefore, the

basis to define d-HDL experimentally lies on its inability or decreased ability to prevent oxidation of LDL. This can be assessed through the use of fluorescence methods by comparison to a “normal” HDL control as, in the absence of preserved HDL function, LDL are oxidized and in turn oxidize a fluorochrome-releasing substrate giving off a fluorescent signal (Navab, Hama et al. 2001, McMahon, Grossman et al. 2006). The inflammatory potential of HDL may also be estimated by assessing monocyte migration in coculture with either aortic or smooth muscle cells in the presence of LDL (Van Lenten, Wagner et al. 2001), although the cell-free assay described originally is more frequently used. Based on this broad methodology, several studies on the role of d-HDL emerged. Van Lenten et al described a series of experiments through which the function of HDL was hindered in the presence of an acute phase-like environment and hypothesized that this could be due to the displacement and/ or exchange of proteins associated with HDL, namely paraoxonase and apoA1 (Van Lenten, Hama et al. 1995). Based on the data obtained from *in vivo* models, which demonstrated that MPO binds to HDL by selectively targeting and oxidizing apoA1, thus leading to an impaired cholesterol efflux and reduced LCAT activation, Undurti et al further explored the full impact of MPO-induced modifications on HDL function (Undurti, Huang et al. 2009). In a series of experiments, they showed that through MPO-catalysed oxidation, not only was there a loss of non-cholesterol efflux actions of HDL, namely of its anti-apoptotic and anti-inflammatory effects, but also that the modified HDL particles actually had a pro-inflammatory action, namely enhanced NF- κ B activation and increased expression of vascular surface adhesion molecules (Undurti, Huang et al. 2009).

Ansell et al reported that d-HDL levels correlated better with the presence of CVD than those of HDL and that treatment with simvastatin led to a decrease in d-HDL (Ansell, Navab et al. 2003). Skaggs et al explored the impact of d-HDL in monocyte function and found that d-HDL caused a small, but functionally significant, alteration of monocyte atherosclerosis-related transcripts, namely by driving a pro-atherogenic phenotype through platelet-derived growth factor receptor β (PDGFR β)-mediated pathways. They showed that in the presence of d-HDL there was an upregulation of PDGFR β , increased PDGFR-dependent chemotaxis and enhanced TNF α secretion which could partially explain the deleterious cardiovascular role of d-HDL (Skaggs, Hahn et al. 2010).

The fact that inflammation appears to be a key factor in determining the shift from “normal” HDL to d-HDL led to exploration of its potential role in chronic inflammatory conditions such as SLE and RA. McMahon et al assessed d-HDL levels in 154 women with SLE and compared them with healthy controls (HC) and patients with RA. They reported that patients with SLE had significantly higher levels of d-HDL than HC and patients with RA and that the proportion of patients with SLE who had d-HDL was also higher. Moreover, patients with

SLE with CAD had significantly higher levels of d-HDL compared with those without CAD. A strong correlation between d-HDL and oxidized LDL levels was also noted (McMahon, Grossman et al. 2006). In a different study, the same group reported on the associations found between d-HDL and IMT thickness and carotid plaque presence. D-HDL was detected in a greater proportion of patients with plaque compared with those without plaque (86.7% vs 40.7% respectively, $p < 0.001$) and the presence of d-HDL was also associated with thicker IMT. After multivariate analysis, the odds ratio for plaque presence and increased IMT in the upper quartile in patients with d-HDL were 16.1 and 2.5 respectively. Although the positive predictive value for a single positive d-HDL and presence of plaque was lower than 30%, the negative predictive value was over 95%. Thus, it was concluded that d-HDL appeared to correlate with increased subclinical atherosclerosis and that it could potentially be used to identify patients with increased atherosclerotic risk (McMahon, Grossman et al. 2009).

Ultimately although there is a pathophysiological rationale implicating the likely involvement of, anti-HDL, anti-apoA1, reduced PON-1 activity and d-HDL in the increased/ accelerated risk of atherosclerosis in SLE, definite evidence remains elusive. At best, they can be considered biomarkers of CVD and their presence is indicative of a greater likelihood of heightened atherosclerotic risk.

One of the goals of my thesis is to investigate whether the presence of anti-HDL and anti-apoA1 has any associations with the actual atherosclerotic burden assessed by vascular US in patients with SLE. I will consider not only IMT and presence/ absence of plaque, but also more detailed analysis of plaque, namely by considering total plaque thickness (TpT), total plaque area (TpA), plaque volume and texture.

1.4: Nitration

1.4.1: Nitric oxide metabolism

Nitric oxide (NO) is a diffusible messenger involved in several physiological processes ranging from vasodilatation, clotting modulation (inhibits platelet aggregation), macrophage-dependent elimination of intracellular parasites, bronchodilation and neurotransmission. NO is synthesized from L-arginine by a family of enzymes called nitric oxide synthases (NOS). There are three identified NOS isoforms: neuronal NOS (nNOS, NOS1) found in neuronal tissue and skeletal muscle; inducible NOS (iNOS, NOS2) which has an ubiquitous distribution; and endothelial NOS (eNOS, NOS3) present in vascular endothelial cells, cardiac myocytes, and in platelets (Oess, Icking et al. 2006). All three NOS isoforms are dependent on calmodulin (CaM) a multifunctional protein which acts as an intermediate messenger transducing calcium signalling by binding calcium ions and modifying its interactions with various target proteins (Chin and Means 2000). When the intracellular calcium levels are high, CaM binds to nNOS and eNOS but it has a tight association with iNOS which is independent of calcium levels. Thus, while the enzymatic activity of nNOS and eNOS is modulated by changes in intracellular calcium levels, leading to transient NO production, iNOS is continuously activated, releasing NO regardless of fluctuations in intracellular calcium levels. iNOS activity is therefore mainly regulated at the gene expression level (Janeway 2005). NOS enzymatic activity is modulated by the presence of its substrates and cofactors within the cell and under certain conditions, NOS may generate superoxide ($O_2^{\cdot-}$) instead of NO, a process referred to as uncoupling (uncoupling of NADPH oxidation and NO synthesis) (Janeway 2005).

Nitric oxide is a highly reactive compound (half-life of a few seconds) which cannot be stored inside the cells producing it. Therefore, it diffuses freely across cell membranes, which in turn make it a unique transient paracrine and autocrine signalling molecule. Its signalling function is dependent on the levels of biosynthesis and local availability. Moreover, NO production by NOS is tightly regulated at transcriptional and translational levels, through co- and posttranslational modifications, and by subcellular localization (Janeway 2005).

Besides the functions described above, NO also plays a pivotal role in oxidation (Khan and Ali 2006). Under physiological circumstances, it can be produced in response to a variety of stimuli and it is essential to the preservation of homeostasis (Radi, Peluffo et al. 2001). However, depending on its concentration and the microenvironment where it occurs, NO production can be potentially harmful. High concentrations of NO can interfere with mitochondrial function, produce DNA damage and induce changes into circulating proteins (Khan and Ali 2006). These changes aren't necessarily mediated directly by NO itself but

through reactive nitrogen species (RNS). As mentioned before, NO is a highly unstable compound which quickly diffuses to the extracellular compartment after being produced, reacting swiftly with other circulating reactive molecules such as oxygen and ROS, chlorine and fluorine. One of the key reactants is superoxide ($O_2^{\cdot-}$) leading to the formation of peroxynitrite (Radi, Peluffo et al. 2001). Peroxynitrite is a short-lived RNS which leads to the reduction of NO bioavailability, meaning that the beneficial physiological effects it would normally produce are impaired. Moreover, peroxynitrite also contributes to the generation of other pro-oxidant species which can produce tissue damage, proimmunogenic DNA modifications and protein dysfunction (Khan and Ali 2006)

Changes in the normal NO metabolism, namely the production of RNS have been identified as key elements in the pathogenesis of a series of diseases such as acute and chronic inflammatory conditions (asthma, ARDS, SLE), ischaemia-reperfusion injury and neurodegenerative diseases such as Alzheimer's disease (Radi, Peluffo et al. 2001, Khan and Ali 2006). The measurement of NO and RNS is notoriously complex due to their high instability in biological systems. A chemiluminescent reaction using ozone ($NO + O_3 \rightarrow NO_2 + O_2 + \text{light}$) is the most commonly used but other methods are also available (Janeway 2005). An indirect way to assess NO production is to measure the elements to which it can induce changes. NO and RNS-mediated protein modifications are stable, frequently irreversible, and can therefore be used a surrogate marker for NO and RNS activity, particularly since they occur during periods of significantly increased NO production.

For the purposes of this thesis, I will focus on NO-induced protein modifications and their biological significance.

1.4.2: Biological significance of protein nitration

Free-radical damage to proteins mediated either by ROS or RNS results mainly in the modification of amino acid residues, side chain cross linking and fragmentation (Khan and Ali 2006). Cysteine (Cys) and the aromatic amino acids such as tyrosine (Ty), phenylalanine and histidine are particularly susceptible to modifications to the protein structure that result in changes in its basic properties, namely charge and conformation. Cys residues have a thiol group (carbon-bonded sulfhydryl: C-SH) which is susceptible to suffer S-nitrosation which is a reversible modification responsible for dynamic, post-translational regulation of most major classes of proteins (Faassen and Vanin 2007). Another protein modification induced by NO is nitrosylation which occurs when NO binds to a transition metal ion like iron or copper contained in proteins such as heme proteins. The nitrosylation of heme proteins like

cytochromes leads to the impairment of their normal enzymatic activity. When this involves ferrous iron, nitrosylation is particularly stable rendering it irreversible. In this thesis I will focus on protein nitration which involves the reaction between peroxynitrite and other RNS and both free and protein bound L-Ty leading to the formation of 3-nitrotyrosine (3-NTy). This post-translational modification is achieved by the substitution of a hydrogen by a nitro (NO_2^-) group in to one of the two equivalent carbon atoms in the ortho position in respect to the OH^- group of the phenolic ring of the Ty residue (Souza, Peluffo et al. 2008). The formation of 3-NTy is quite stable and was previously thought to be irreversible. However, recent studies suggest that it can potentially be reversible through the action of the enzyme denitrase which could mean that protein nitration could play a regulatory role (Irie, Saeki et al. 2003). However, it is unlikely that this would be the case when the NO production is greatly amplified such as found in inflammatory conditions. Therefore, the nitration of proteins is viewed generally as harmful as it usually leads to a loss of protein function.

In SLE, there is increasing evidence suggesting that NO can play a role in its pathogenesis (Khan and Ali 2006). The presence of chronic, sustained inflammation may lead to persistently increased production of NO and consequently of RNS, which can in turn produce the effects previously discussed. Murine models of SLE have greater NO levels than control mice and the systemic blockade of NO production leads to an improvement of disease activity (Khan and Ali 2006). In patients with SLE, the serum nitrite/ nitrate ratio which can be used as a surrogate marker for NO production was found to be associated with disease activity and anti-dsDNA levels (Khan and Ali 2006). The source for the increase in NO levels in SLE could potentially be attributed to enhanced NOS activity in the endothelial cells (due to vasculitis) and keratinocytes (due to skin involvement).

As previously stated, protein-bound nitrotyrosine is a post translational modification induced by NO-derived oxidants which can be found abundantly not only in mouse, but also in human atheroma (Leeuwenburgh, Hardy et al. 1997, Parastatidis, Thomson et al. 2007). In patients with CVD, 10-100 μmol of 3-NTy per mol of Ty were identified in the serum, however tissue concentrations of 3-NTy were found to be even higher. When looking at the 3-NTy content of LDL found in atherosclerotic plaques, it was found to be almost 90 times greater than peripheral concentrations (Leeuwenburgh, Hardy et al. 1997). Therefore, there is increasing evidence that raised 3-NTy levels may correlate with CVD and could potentially be considered as a risk factor for CVD (Shishehbor, Aviles et al. 2003).

Moreover, several studies suggest that high systemic levels of nitrotyrosine may be associated with increased CVD risk and may even be modulated by lipid-lowering agents like statins (Shishehbor, Aviles et al. 2003, Shishehbor, Brennan et al. 2003, Shishehbor,

Patel et al. 2006), further strengthening the hypothesis that protein nitration could be a biomarker for increased atherosclerotic burden.

In addition to the potential role of generalized protein nitration associated with sustained chronic inflammation, the nitration of specific targets could have a particular relevance. ApoA1, the major component of HDL is a potential target for nitration which could in turn be associated with changes on apoA1 or HDL function.

It has been shown that apoA1, modified by tyrosine nitration and chlorination, in both plasma and artery walls was greatly increased in patients with CAD compared with healthy controls (Marsche, Hammer et al. 2002, Parastatidis, Thomson et al. 2007).

It has been shown that apoA1 can be nitrated and that MPO may be the main driver inducing this modification (Zheng, Nukuna et al. 2004, Zheng, Settle et al. 2005). Zheng et al proposed that apoA1 could be a selective target for MPO-catalysed oxidative modification in human atheroma nitration. Furthermore, they suggested that MPO-induced nitration of apoA1 could be a potential mechanism for generation of a proatherogenic dysfunctional form of HDL (Zheng, Nukuna et al. 2004). The same group later reported on their findings regarding the mapping of the different tyrosine residues in the apoA1 molecule that can potentially be nitrated. They reported that in human atheroma, the MPO-induced nitration of apoA1 occurs preferentially on Tyr-192 and Tyr-166 and that these changes were associated with impaired ABCA1-dependent cholesterol efflux from macrophages (Zheng, Settle et al. 2005). Vasquez et al also reported on the impact of apoA1 nitration on impaired ABCA1-mediated cholesterol efflux in obese women and suggested that increased apoA1 nitration could be a biomarker of increased oxidative stress even in the absence of diabetes or dyslipidemia (Vazquez, Sethi et al. 2012). Finally, Gugliucci et al reported on a case control study of 15 patients with type 2 diabetes and 15 age and sex-matched controls comparing the levels of PON-1 activity and nitrated apoA1 and found that nitrated apoA1 predicted 64% of PON-1 activity. Moreover, diabetic patients had significantly lower PON-1 activity and increased nitrated apoA1 compared with healthy controls in the absence of statistically significant differences in the lipid profile (Gugliucci, Hermo et al. 2006). This finding raises the possibility that the presence of enhanced nitrosative stress could lead to apoA1 modifications and consequently impairment of HDL function due to reduced PON-1 activity.

In summary, there is evidence to support the presence of a sustained inflammatory state in SLE which can lead to systemic endothelial activation leading to the production of large amounts of NO. Nitration of proteins is a stable NO-induced change which can act as a surrogate marker for production of NO and consequently the extent of endothelial activation

which could lead to endothelial dysfunction over time. Considering that endothelial dysfunction is the first event in the atherosclerotic process, assessing nitration levels in patients with SLE could be useful as it will allow for an estimation of the severity of endothelial insult. Moreover, since it has been shown that patients with SLE have increased CVD and may have an accelerated atherosclerotic process, the presence of raised nitration levels could potentially correlate with the presence of subclinical CVD thus becoming useful as CVD-risk biomarker.

In my research, I selected three targets for nitration: nucleosomes, albumin and apoA1. I chose nucleosomes because both nucleosomes and anti-NCS levels have been found to be elevated in patients with SLE and, as previously discussed, considering their potential role in the pathogenesis of SLE, measuring the levels of nitrated nucleosomes (NN) could prove to be useful. The second nitration target I selected was albumin because it is a ubiquitous protein in the serum and nitrated albumin (NA) could therefore be used as an overall marker of nitrate stress. Finally, the choice of apolipoprotein A1 stemmed from the notion that if apoA 1 has a potentially CVD protective role, as it is the main structural component of HDL, the presence of modifications could hinder its function thus predisposing to increased atherosclerotic burden. In order to investigate this hypothesis, I measured total and nitrated ApoA1 levels in a cohort of patients who also underwent vascular US scanning, thus enabling me to assess the correlation between nitrated ApoA1 levels and the presence of atherosclerotic plaques.

Moreover, measuring both NN and nitrated apoA1 may provide a means to assess nitration and allow an assessment of whether the nitration of nucleosomes and apoA1 correlates with anti-NCS and anti-ApoA1 levels which could be a potential mechanism for rendering them more antigenic.

My hypothesis is that levels of nitration (measured through NN, NA and nitrated ApoA1) in patients with SLE may be elevated, particularly during flares of disease activity. Furthermore, I hypothesize that the immunological profile of the patients, such as the ENA status, as well as their treatment could influence the levels of nitration. To test these hypotheses, I measured NN and NA levels in 397 samples taken at different time-points of varying disease activity in a population of 49 patients with SLE.

1.5: Vascular Ultrasound

1.5.1: Basic Principles in Vascular Ultrasound

The term “ultrasound” (US) describes longitudinal pressure waves within a propagating medium, which oscillate at frequencies above the approximate threshold of human hearing, customarily set at 20,000 Hertz (Hz) or 20 kHz ($K = 10^3$). Sound waves are longitudinal mechanical vibrations or pressure mechanical waves caused by the oscillations of particles in a medium leading in part to the transference or propagation of some of the energy through the medium and can be classified as a form of non-ionising radiation. The medium is essential for the sound wave to be able to propagate contrary to self-propagating electromagnetic radiation which can move without a supporting medium. In fact, the sound wave is the medium in a state of oscillation and transferring energy (Venables 2011).

Sound waves have certain physical characteristics that can be used to describe them. These include frequency, period, wavelength, propagation speed, amplitude, and intensity. Frequency, period, amplitude, and intensity are determined by the sound source. Propagation speed is determined by the medium (acoustic impedance) and both the source and the medium determine wavelength (Venables 2011).

The types of interactions between ultrasound and tissue are similar to those of light, and include the following *reflection*, *refraction*, *scattering*, *diffraction*, *divergence*, *interference*, and *absorption*. With the exception of interference which can either increase or decrease intensity, all these interactions reduce the intensity of the beam (*attenuation*)(Hutchison 2011, Venables 2011).

In Medicine, the most common application of US is in imaging known as ultrasonography. It has become possible, in vascular ultrasonography, to assess not only the arterial wall structure, but also the changes it undergoes as a result of atherosclerosis. It can also provide information on blood flow patterns. Therefore ultrasound is a key player in the evaluation of vascular disease.

Images produced for diagnostic ultrasound result from the reflected portion of a sound beam. If a sound beam is directed at right angles, called normal incidence, to a large interface (greater than the sound beam width), the beam will be partially reflected back towards the sound source. The angle of reflection of the sound beam is equal to its angle of incidence. To obtain maximum detection of the reflected sound, the transducer (which sends and receives sound) must be positioned so that the sound beam strikes the interface perpendicularly. The percentage of beam reflected at tissue interfaces depends on the tissue's acoustic impedance and the beam's angle of incidence. The acoustic impedance is

a measure of the resistance to sound passing through a medium (expressed in $\text{kg} / \text{m}^2\text{-sec}$). The higher the density of the medium, the higher the acoustic impedance. It is primarily the change in the acoustic impedance at a biological interface that makes it possible to visualise soft tissue structures with an ultrasonic beam. The amount of reflection is determined by the angle of incidence between the sound beam and the reflecting surface. The higher the angle of incidence, that is the closer it is to a 90° angle, the less the amount of reflected sound. The ideal angle of incidence is perpendicular to the structure that is being assessed (Hutchison 2011).

1.5.1.1: US transducers

A transducer is a device that can convert one form of energy into another. Ultrasonic transducers are used to convert an electrical signal into ultrasonic energy that can be transmitted into tissues, and to convert ultrasonic energy reflected back from the tissues into an electric signal. Ceramic material is used for the construction of piezoelectric crystals, which have the ability to change shape when a voltage is applied across it, thus the vibration of these crystals is used as a source of ultrasound energy.

Transducers consist of an array of closely spaced piezoelectric elements that can be excited individually or in groups to produce ultrasound beams, since each element has its own electrical connection to the ultrasound instrument. In addition, echo signals detected by individual elements are amplified separately before being combined into one signal for each reflector (Venables 2011).

The imaging of the carotid and femoral arterial bifurcations, and the lower limb arterial tree for this thesis was performed with a broad bandwidth linear array transducer. A 3D probe was used to image carotid arteries when plaque was found.

In diagnostic ultrasound, reflection of the sound beam is of primary interest. Therefore transducers are used in pulsed mode. A short pulse of ultrasound is produced in a known direction and allowed to propagate into a medium. Any reflections that occur along the path of the pulse cause echoes to propagate back to the transducer. In pulsed mode the transducer is now capable of receiving these echoes as signals because it is no longer transmitting. The signals from distinct, separate boundaries are themselves distinct, and separate, since reflection only occurs while the pulse propagates across any particular boundary. Displaying any sequence of echo signals in time as they return means that the time gap between the initial transmission pulse and the reception of any particular echo signal may be measured. If the velocity of sound in the medium is known, then a measured time gap for any echo signal may be used to calculate the distance away from the probe of the reflecting boundary that caused the echo signal. This results in spatial information,

known as echo ranging or the pulse-echo principle, the basis for imaging. This concept forms the basis for most ultrasonic scanning modes (Lutz, Buscarini et al. 2011).

The majority of US devices have a large array of technical options and features which fall beyond the scope of this thesis. Briefly, those most commonly used in vascular US are the “Freeze”, the “Zoom”, the “Callipers” and the “Gain” function. The “Freeze” function allows for the image to be held (frozen) on the screen thus enabling measurements to be made and images recorded. The “Zoom” will magnify certain areas of interest in the US image increasing the detail of a certain view. The “Callipers” can be used to make length measurements by selecting the start and finish point of a certain region and it is usually used in conjunction with other functions such as “Freeze” and/ or “Zoom”. Finally, the “Gain” function allows for brightness control as it amplifies the electronic signal received by the transducer thus regulating the strength of the echoes being received (the greater the gain defined, the greater will be the brightness of the image obtained).

1.5.1.2: Brief overview of Ultrasonic Scanning Modes

B-mode ultrasound

B-mode refers to brightness mode. The object of B-mode is to produce a two-dimensional sectional image through the body. The amplitude of the signals, received from the internal structures (acoustic boundaries) which form the image, are represented as a series of dots of varying brightness. The B-mode image is built up from these dots line by line. On average 100 or more B-mode lines are needed per one image. Since signals are obtained from many directions, a special arm is required to determine the location of pulse-echo information (Lutz, Buscarini et al. 2011).

M-mode ultrasound

Referred to as motion-mode, this is in essence a strip chart recording of the changing position of the B-mode dots. This mode displays changes in the position of reflecting structures with respect to time, that is reflector depth on one axis and time on an orthogonal axis. M-mode is commonly used in the evaluation of rapid motion, including heart valves, cardiac chambers and vessel wall motion (Lutz, Buscarini et al. 2011).

Real-time B-mode ultrasound

Dynamic or real time imaging provides continuous data acquisition at a rate sufficient to give the impression of instantaneous motion of moving structures. It is the rapid sequential display of static ultrasound images taken in a short period of time and viewed as they are

acquired resulting in an impression of motion. Depending on the real time scanning equipment, 15 up to 120 images per second can be produced. This is currently what is used in ultrasound imaging.

Doppler Ultrasound

The Doppler Effect is a change in the perceived frequency of sound emitted by a moving source. The resultant shift in the Doppler frequency is related to the velocity of the reflectors (Polak, Bajakian et al. 1992) and is named after the physicist Christian Doppler (1803-1853), who discovered it in 1843.

There are two Doppler techniques to measure blood flow frequency shifts, differing in the way of presenting and interpreting the results: the spectral Doppler technique and colour Doppler flow imaging. In the case of spectral Doppler, a single sound beam cuts the vessel, and flow velocity is detected only along this sound beam direction and displayed as a spectral distribution. In the continuous wave (CW) Doppler mode this measurement is carried out over the whole depth of the sound beam, while in pulsed wave (PW) Doppler mode only echo signals from a sample volume in a pre-selected depth are analysed (Rubens and Grant 2011).

The relationship between peak systolic frequency shift and lumen narrowing of an artery is the basic principle that permits the use of Doppler ultrasound to detect and grade significant stenosis of the carotid and peripheral arteries.

In this study, I used only colour and power Doppler and therefore I will only describe these two methods.

Colour Doppler

Doppler colour flow imaging was first introduced for echocardiography instrumentation in 1984, for peripheral vascular imaging in 1986, and for general radiology use in 1987. Doppler shifts from moving particles are shown in colour, providing flow information throughout the grey scale field. These colour flow systems detect and process the amplitude, phase, and frequency of the returning echoes (Rubin and Adler 1993). With the use of the pulse echo imaging principle, colour Doppler denotes both velocity and flow direction in colour codes. Multiple sample volumes along each line of the image determine direction and average flow rate in each part of the real time image. Flow is displayed by encoding in colour an estimate of the mean Doppler frequency shift at a particular position.

A different colour is assigned to represent flow direction. Colour intensity indicates the degree of the frequency shift and the magnitude of the velocity of blood flow. In vascular ultrasound the colour scale most frequently used is red to mark flow direction towards the probe and blue to mark direction away from the probe. However, the operator can select other colour scales as provided by many ultrasound scanners (Hutchison 2011).

Colour Doppler has some limitations such as aliasing. With pulsed ultrasound, there is an upper limit of the Doppler shift which can be displayed, the Nyquist limit, defined as the pulse repetition frequency/2. When high velocity blood flow is present, Doppler shifts above the Nyquist limit are generated, leading to the appearance of bright, turbulent-appearing flow in colour Doppler, i.e. aliasing. Aliasing can be a problem as it distorts the direction and velocity information in Doppler imaging. If the PRF is set too low, this will limit the maximum frequency that can be displayed correctly, causing a “wrap around” of colour almost as if the flow was in the opposite direction. One must also be aware that setting too high a PRF could prevent the detection of low velocities. Colour Doppler is also angle dependent and the homogeneous representation of colour velocities is extremely dependent on the proper angle being used. Colour flow imaging can pinpoint specific areas of abnormal flow for further interrogation, but interpretation from the colour image alone in terms of velocities, turbulence and flow patterns should be treated with caution until one can understand and allow for all possible artefacts inherent in the colour image (Hutchison 2011).

Power Doppler

As previously stated, colour Doppler imaging shows flow by encoding in colour an estimate of the mean Doppler frequency shift. In contrast, power Doppler encodes the power in the Doppler signal in colour by using the amplitude information from the colour Doppler signal. This feature allows the visualisation of flow in small vessels that would otherwise be difficult to detect (Martinoli, Pretolesi et al. 1998, Hudson-Dixon, Long et al. 1999). Power Doppler provides a perfusion-type display of blood flow and is three to five times more sensitive than colour Doppler (Rubin and Adler 1993).

The primary advantage of power Doppler is its increased sensitivity for the detection of low perfusion echoes. The amplitude information acquired by this mode is independent of red cell velocity and the direction of blood flow. Power Doppler does not rely on frequency shift information, is not dependent on beam angle, and is free from aliasing. This is ideal for imaging tortuous vessels. This technique also provides edge definition for plaques.

The main disadvantages of this technique include its failure to provide directional or velocity information and therefore all vessels in the path of the beam are represented in the same

colour mode. Excessive motion can seriously affect the image and therefore it is not suited to rapid scanning along vessels (Rubens and Grant 2011).

1.5.1.3: Brief anatomical overview of the carotid and femoral territories

In human anatomy, the principal arteries supplying to the head and neck region are the 2 common carotids (CCA). The right CCA begins at the bifurcation of the innominate artery whilst the left CCA springs directly from the aortic arch. Although there is a great degree of anatomical variation, around the jaw level, both CCAs balloon out forming the carotid bulb and then divide into their main branches, the external carotid artery (ECA) and internal carotid artery (ICA) (Figure 2). The first supplies the exterior of the head and neck as well the face and eye region, whereas the latter supplies the brain and intra-orbital cavity by joining its contra-lateral in a circle of arteries located on the base of the skull (Circle of Willis) thus regulating the intra-cranial circulation. Similarly, the common femoral artery (CFA) divides into two main arteries at groin level, the superficial and the deep femoral artery. The first runs along the inside of the thigh and divides into the calf arteries in the popliteal region whereas the latter divides proximally, eliciting the smaller arteries that supply the muscles of the thigh and buttock (Gray, Standring et al. 2005).

Generally, the CCA is a relatively long and straight segment and therefore the blood flow is usually laminar, minimizing shear-stress forces on the arterial wall. Conversely, the bulb region is characterized by turbulent flow due to the presence of an anatomical divide. As will be discussed later, the change in blood flow increases the likelihood of endothelial damage and consequently of enhanced cholesterol deposit and plaque formation. The CFA bifurcation differs from that of the CCA as it does not dilate before dividing. Nonetheless, there the blood flow also becomes more turbulent in the region, increasing the likelihood of plaque formation.

A simple schematic of the anatomical aspect of the CCA and CFA bifurcations is showed in Figure 4.

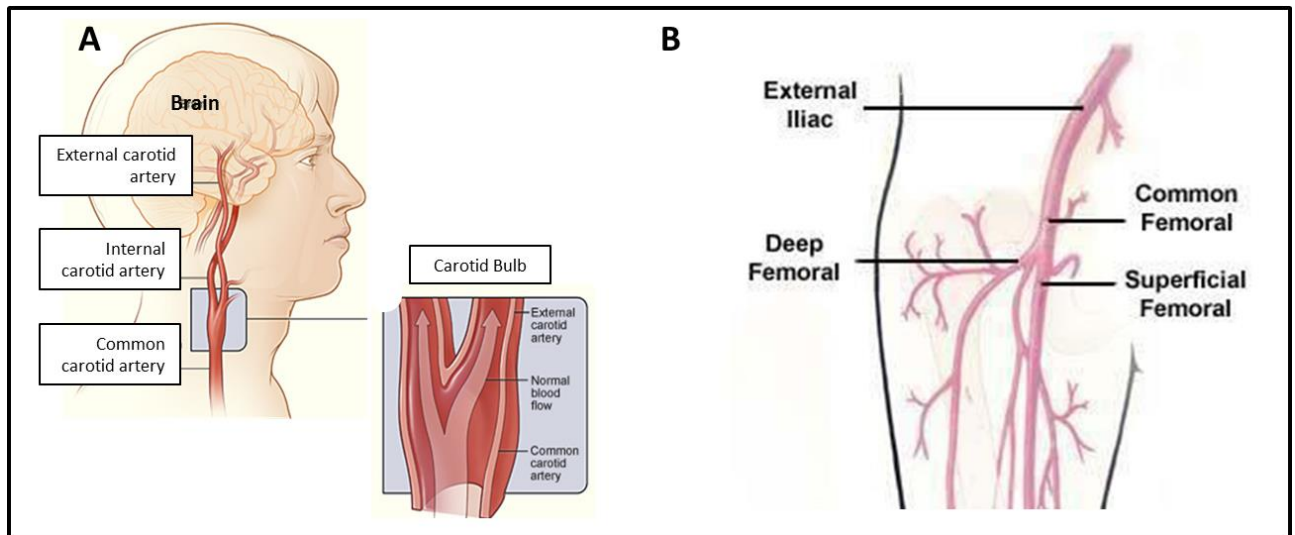


Figure 4: Overview of the carotid and femoral territories: A) Common carotid artery (CCA) and its two main branches, the internal and external carotid arteries. The carotid bulb is showed in detail. B) Common femoral artery (CFA) division into deep and superficial femoral arteries.

Atherosclerosis may be assessed in various ways and quantifying it is extremely useful for determining the extent of its burden and evolution over time as it allows for risk stratification and determining response to treatment. Risk stratification is particularly important in CVD as it impacts on patient management, namely the type and intensity of medical treatment, ideally allowing for primary prevention. A number of well-known risk factors such as age, sex, serum cholesterol, smoking and blood pressure can be used in a series of risk-stratification algorithms such as the Framingham risk score (D'Agostino, Vasan et al. 2008). Unfortunately, although these systems may be useful, they are imperfect and, particularly in the context of SLE, they can grossly underestimate the real CVD risk as previously discussed. Other imaging methods have been developed to increase the sensitivity of risk prediction such as coronary calcium scoring (Alexopoulos and Raggi 2009) but the radiation load necessary to such tests is substantial and cannot be neglected. Another limitation of some of these methods is their low predictive value, i.e. they fail to increase the area under the curve of receiver-operator curves for prediction of events based on traditional risk factors. US is a completely non-invasive, radiation-free imaging method which allows for accurate assessment of superficial vessels such as the carotids and femorals, rendering it a particularly appealing technique for assessing asymptomatic patients. US techniques aiming to quantify the atherosclerosis-related burden have also been developed for the purpose of risk stratification. They include one-dimensional (B-mode) measurement of IMT and plaque thickness (Ebrahim, Papacosta et al. 1999, Lorenz, von Kegler et al. 2006, Simon, Megnien et al. 2010), 2D plaque area and 3D measurement of plaque and vessel wall volume. The measurement of both plaque area and volume has greatly increased the predictive value of vascular ultrasound scanning for cardiovascular events among patients free of CVD at baseline. A correlation was found between carotid plaque and IMT thickening with the occurrence of both stroke and myocardial infarction (Bots, Hoes et al. 1997, O'Leary, Polak et al. 1999, Lorenz, Markus et al. 2007) thus highlighting the utility and consistency of using non-invasive measurements to assess risk factors based on vessel wall biology. The combination of US data including both IMT and plaque assessment allows for a more accurate evaluation of the *actual* "vascular health". Therefore, data from carotid US can potentially change the risk stratification obtained through traditional risk stratification algorithms which is of particular importance in patients for which those are notoriously flawed such as in SLE.

1.5.1.4: Normalization of US vascular images

With the generalization of vascular US use since the 1980's, the need to characterize US findings, specifically plaque images, emerged and with it the realization of its subjectivity and difficulty of comparing results between different operators and different US equipment. A

more objective assessment of plaque echodensity was achieved with the use of computer-assisted measurements of digitalized B-mode images (el-Barghouty, Nicolaides et al. 1996). The software initially used was the commercially available Adobe Photoshop TM (Adobe Systems, San Jose CA) but later other, dedicated software like the programme I used in the course of my research were developed.

The underlying concept for this type of image processing is the fact that the black and white images obtained in B-mode are composed of a spectrum of pixels with different values on a computer gray scale which usually ranges from 0 (absolute black) to 255 (absolute white). Furthermore, different colour pixels appeared with varying frequencies enabling the construction of a histogram which revealed a skewed distribution. For that reason, the median value of the gray scale was used as the measurement of the overall echodensity for any given image, thus defined as the grey scale median (GSM). The notion that every US image can be translated into a grey scale whose extremes can be set to specific values, is the basis for image normalization (Biasi, Sampaolo et al. 1999).

In order to allow for comparability, each individual image can be normalized by means of digital image processing using blood and adventitia as the two reference extremes of the spectrum: blood (black) defined as the zero value (absolute echolucence) and adventitia (white) as the maximum value (absolute echogenicity). Thus, the brightness of all pixels in the image, namely those present in the plaque, could be represented on a linear scale defined by these two reference values (Elatrozy, Nicolaides et al. 1998). The choice of these two references was made based on the repeated analysis of high quality images by two experienced independent observers and subsequently measuring the GSM value of blood (0-5) and adventitia (180-190). The distinction between adventitia and other highly echogenic media such as bone/ calcification was ensured as the latter usually present higher values (>250) (Griffin, Nicolaides et al. 2007).

Moreover, the definition of the image (number of pixels per mm) can also be adjusted to a set value, further limiting the differences attributable to method/ equipment use. Thus, the process of normalization allows for the differences between images obtained in different conditions such as equipment or patient characteristics (body fat, muscle mass) to be attenuated by establishing a similar colour range and definition.

It can be argued that the US images thus processed do not correspond to the actual image obtained when scanning a patient. However, the results obtained from several large scale reproducibility studies have shown that normalization does not change the characteristics of US images obtained, namely those of atherosclerotic plaque and actually improves variability, by attenuating differences related with the US equipment used and type of image

gain considered (Biasi, Sampaolo et al. 1999, Sabetai, Tegos et al. 2000, Tegos, Sabetai et al. 2000) .

Tegos et al performed a thorough assessment of the comparability of the US characteristics of carotid plaques, i.e. the reproducibility of before and after plaque image normalization. Initially, a 3-step experimental phase was done by assessing 26 carotid plaques from 16 patients: firstly, all plaques were scanned by three independent operators using the same protocol and equipment and individual pre and post-normalization GSM measurements; secondly, all plaques were assessed in a similar manner by the same operator using two different US devices and finally, all plaques were assessed by the same operator, using the same equipment but with four different types of image settings on the US i.e. varying levels of image gain (described in the 1.5.1.1 section: "US transducers"). When comparing the distributions of GSM in non-normalized and normalized images, no statistically significant differences were found. After this experimental phase, an evaluation of the clinical application of the examination was done by assessing 315 patients with a total of 419 carotid bifurcation plaques producing greater than 50% stenosis on duplex examination which were treated as an independent case (Tegos, Sabetai et al. 2000). Plaques were further divided into asymptomatic (n= 273) and symptomatic (n= 146) and comparison of the GSM between these 2 groups showed a statistically significant difference. Although this difference was apparent for the pre-normalized images (GSM symptomatic vs. asymptomatic: 10.5 vs. 20), it became even more pronounced after normalization (GSM symptomatic vs asymptomatic: 8 vs. 28). These findings suggest that plaque image normalization is a reproducible method which reduces the variability of the US tissue characteristics of carotid plaque attributable to different degrees of US gain and different US equipment. Moreover, it demonstrated its clinical usefulness by emphasising the differences between two clinically distinct groups (symptomatic vs. asymptomatic) in terms of plaque echostructure (Tegos, Sabetai et al. 2000).

El-Barghouty et al. assessed 190 patients with 329 carotid plaques producing an over 50% stenosis determining their echogenicity on vascular US using computerised measurements of the GSM. Plaque heterogeneity was determined by comparing the GSM between the most echogenic and most echolucent areas within each plaque (heterogeneity index). The presence of ipsilateral cerebral infarction was assessed by CT brain scan. It was found that cerebral infarction was more common in patients with echolucent plaques, i.e. plaques with lower GSM, than echogenic plaques (GSM 29.7 vs. 37.8, $p < 0.01$). Furthermore, plaques with $GSM \leq 32$ were significantly associated with a higher incidence of cerebral infarction compared to those above this level, regardless of the presence or absence of symptoms. Thus, the authors showed that the identification of high risk carotid plaques could be done by

using GSM in both symptomatic and asymptomatic patients, as echolucent plaques (i.e. $\text{GSM} < 32$) were five times more likely to have silent ischaemic stroke documented by CT scan than those whose GSM was greater (Elatrozy, Nicolaides et al. 1998).

The paper of Biasi et al. (1999) echoed these findings (Biasi, Sampaolo et al. 1999). They selected 186 patients with 232 asymptomatic carotid plaques causing $\geq 60\%$ stenosis. Patients with conditions predisposing to cardioembolic cerebrovascular disease (atrial fibrillation, congestive heart failure) were excluded to limit potential confounding factors. Upon recruitment, CT brain scans were performed and the presence of ischaemic lesions in the middle and anterior cerebral artery territory was noted. In the first part of the study, the pre and post-image normalization variability of 26 carotid bifurcation plaques (19 patients) using different storage media, different US probes and different degrees of magnification was assessed. Significant differences in plaque echogenicity were found depending on the type of image storage method used, but these were eliminated after image normalization. Similarly, although significant differences were found in plaque images taken with different linear array probes and when different magnifications were used, these were eliminated after image normalization. The second part of the study focused on the clinical usefulness of normalization which was illustrated by the fact that a significant difference between asymptomatic plaque echogenicity and the presence of non-lacunar CT-demonstrated brain infarcts only became apparent after image normalization (Biasi, Sampaolo et al. 1999).

In conclusion, the use of image normalization enables the quantification of carotid plaque echogenicity and to overcome the subjectivity of pre-existing classifications by using a simple, computer-assisted method. Furthermore, it decreases variability associated with different types of US equipment and may improve inter-observer variability. Finally, the normalization of plaque images may improve the strength of the correlation between the presence of plaque and the risk of ischaemic infarcts, not only in symptomatic but more importantly in asymptomatic patients.

1.5.2: Importance of Vascular US in Cardiovascular Disease Assessment

1.5.2.1: IMT Measurement

As shown in Figure 5, histologically, the wall of elastic arteries such as the aorta and the common carotid is formed of three main layers: the intima, the media and the adventitia. The intima is the innermost, composed of a single layer of flattened endothelial cells together with a supporting layer of elastin rich collagen. Also in the intima, fibroblasts and myointimal cells can be found. It is these cells which can accumulate lipids, ultimately leading to the

thickening of the intima layer which is one of the first signs of atherosclerosis. The media is a broader, elastic layer formed by concentric fenestrated sheets of elastin and collagen and a small amount of smooth muscle fibres. Finally, the adventitia is relatively thin connective tissue layer predominantly formed by fibroblasts and collagen and elastin fibres. This particular morphology allows enough elasticity to accommodate high pressure blood flow as well as preventing extensive stretching during systole. Blood vessels supplying the adventitia and outer media are also present within the adventitia (*vasa vasorum*). A transverse view of a transverse histological cut of a common carotid artery is shown on Figure 5-A.

Other types of arteries such as the muscular (common femoral) and the smaller arterioles have slightly different morphology, rendering it impossible to accurately identify and measure the IMT. Therefore, whenever referring to the IMT, I will be doing so in relation to the common carotid arteries.

B-mode US has allowed direct visualization of both the lumen and the vessel wall of superficial arteries (Pignoli, Tremoli et al. 1986). By the use of this technique it is possible to identify on the common carotid artery wall, two echogenic bands separated by a relatively anechoic space as shown on Figure 5-B.

These lines were shown to be generated by the blood-intima and media-adventitia interfaces, respectively (Baldassarre, Werba et al. 1994). The distance between these two lines was defined as the intima-media thickness (IMT) and importantly it has been shown to directly correlate with histology (Gamble, Beaumont et al. 1993, Wong, Edelstein et al. 1993).

The appearance of the intima-media junction on US can be described using a qualitative scale, the AUS score which ranges from 0 to 10 as shown on Figure 6.

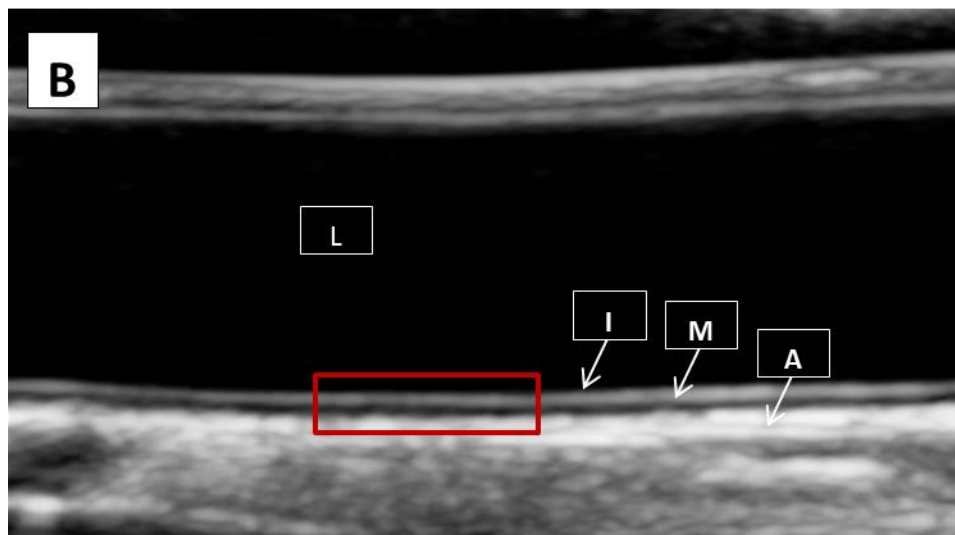
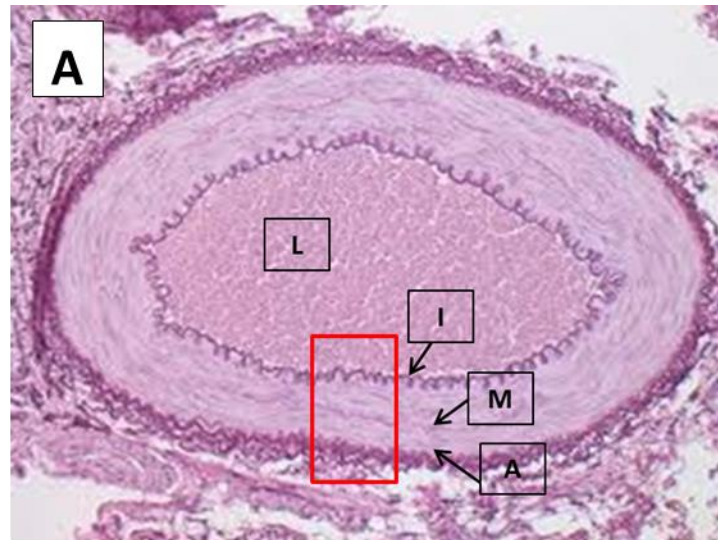
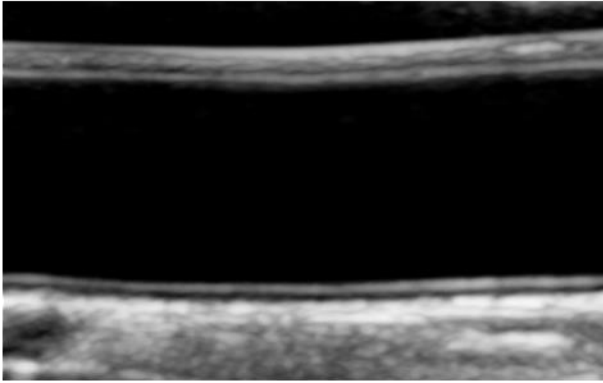
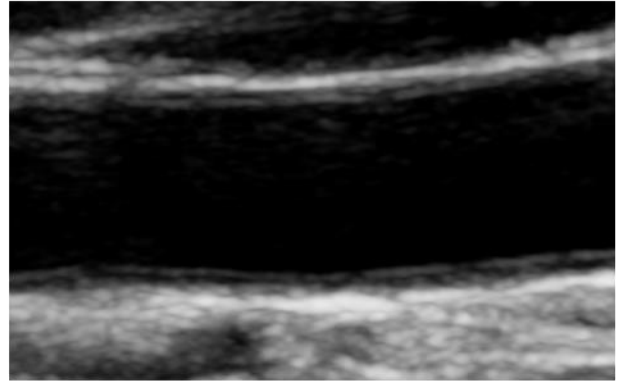


Figure 5: IMT views - Cross section of a common carotid artery in a transverse histological cut (A) and on a B-mode longitudinal view (B). The red rectangle highlights the intima-media complex on both images (arterial lumen-L; intima-I; media-M and adventitia-A).

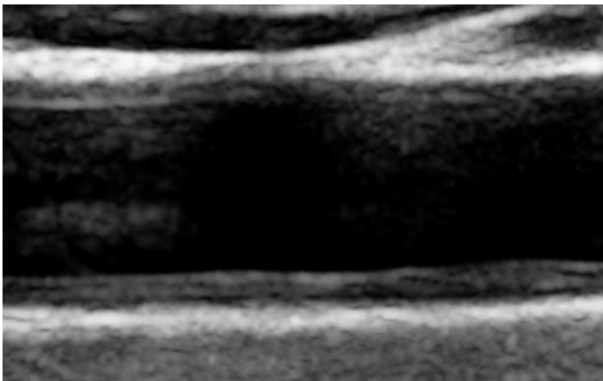
A (AUS 0)



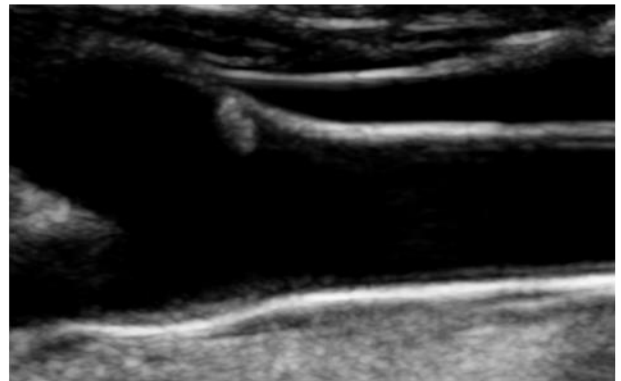
B (AUS 2)



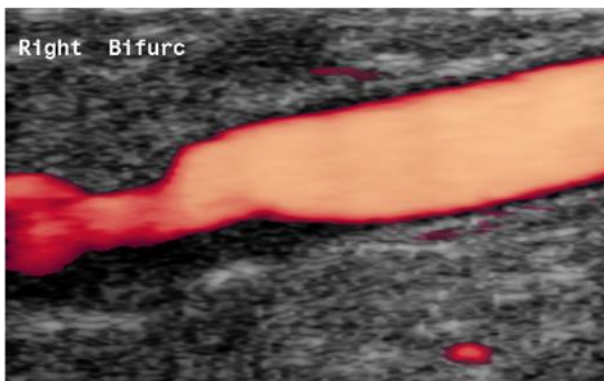
C (AUS 4)



D (AUS 6)



E (AUS 8)



F (AUS 10)

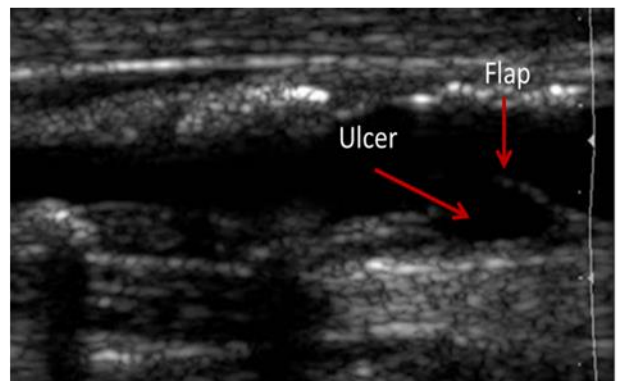


Figure 6: Arterial ultrasound score (AUS): The AUS describes increasing changes to the IMT as visualized in US, ranging from 0 (completely normal IMT) to 10 (symptomatic plaque).

A score of 0 represents normal IMT which appears as a straight, uniform thin line on US as the echoes are reflected back at a 90° angle from a completely normal endothelium (Figure 6A). In score 2, small gaps in the IMT correspond histologically to the presence of lipid streaks. Lipid deposits are echolucent, i.e. they permit the passage of ultrasonic waves without echoes and therefore the representative areas appear black on US (“acoustic holes”). Thus, the presence of lipid streaks in the endothelium leads to a scattering of the echoes and consequently the wavy appearance of the IMT (Figure 6B). Score 4 describes the stage when IMT thickening can be observed ($>0.1\text{cm}$) whereas score 6 corresponds to the presence of a plaque occupying less than 50% of the total arterial lumen (Figures 6C and 6D respectively). Finally, scores 8 and 10 describe the presence of plaques that either occupy $>50\%$ of the arterial lumen (Figure 6E) or are symptomatic (Figure 6F) (Belcaro, Barsotti et al. 1991, Belcaro, Laurora et al. 1993). The increase in IMT is the result of intimal thickening, smooth muscle hyperplasia and intimal fibrocellular hypertrophy. These features are traditionally seen in arteriosclerosis which is mainly associated with age-related sclerosis and systemic hypertension (Roman, Saba et al. 1992).

The most relevant clinical correlate between the intima-media junction and CVD risk is the IMT. The first studies of IMT measurement were developed by Bond *et al.* (Bond, Wilmoth et al. 1985) through US studies performed in monkey models. Subsequently the technique was adapted and evolved to be used in human studies (Bond, Wilmoth et al. 1989, Baldassarre, Amato et al. 2007).

Earlier clinical studies suggested that an increase in the IMT of extra-cranial carotid arteries correlated with both carotid and coronary atherosclerosis and paralleled the significance of traditional risk factors (Baldassarre, Veglia et al. 2000, Amato, Montorsi et al. 2007, Baldassarre, Amato et al. 2007, Touboul, Hernandez-Hernandez et al. 2007). It was therefore, hypothesised that the measurement of carotid IMT could be used as a surrogate marker of atherosclerosis (Espeland, O'Leary D et al. 2005) as well as a predictor of cardiovascular events (i.e., the thicker the IMT the higher the rate of myocardial infarction or stroke (O'Leary, Polak et al. 1999). Interestingly, IMT studies in children and young adults have shown an association between increased carotid IMT and exposure to traditional risk factors such as high LDL and t-cholesterol (Li, Chen et al. 2003, Stein, Fraizer et al. 2004, Wiegman, de Groot et al. 2004). Notwithstanding this association between IMT and traditional CVD risk factors, it appears that the individual genetic background may also play an important role (Lanktree, Hegele et al. 2009). In fact, many of the factors that influence IMT can be considered, at least partially, to be genetically determined, namely the presence of familial hypercholesterolemia, systolic blood pressure and type 1 diabetes. These factors

are also closely related with the development of arteriosclerosis which is defined as arterial sclerosis (smooth muscle hyperplasia and intimal fibrocellular hypertrophy) which is mainly due to chronic pressure overload against the arterial wall due to increased blood pressure. Thus, the presence of arteriosclerosis could be the underlying cause for IMT thickening (Roman, Saba et al. 1992).

This notion that there is a strong genetic determination for IMT thickness is supported by studies done in children, showing that those with hypercholesterolemia and diabetes had increased IMT compared to healthy controls (Jarvisalo, Jartti et al. 2001).

Despite the widespread use of IMT measurement in clinical trials (Hurst, Ng et al. 2007) recent data appear to suggest that its predictive role is not that strong. Lorenz et al. (Lorenz, Markus et al. 2007) reviewed and summarized the data from more than 37,000 patients and reported that the age and sex-adjusted overall estimates of the relative risk of myocardial infarction were 1.26 (95% CI, 1.21-1.30) per standard deviation of the common carotid IMT difference and 1.15 (95% CI, 1.12-1.17) per 0.10mm of the common carotid artery IMT difference. The age and sex-adjusted relative risks of stroke were 1.32 (95% CI, 1.27-1.38) per standard deviation of common carotid IMT difference and 1.18 (95% CI 1.16-1.21) per 0.1mm common carotid artery IMT difference. The conclusions were that the IMT appeared to be a stronger predictor of stroke than of MI. The main sources of heterogeneity were related to age distribution, definition of the carotid segment and the type of IMT measurement protocol used. This report, although not denying the concept that IMT may be a surrogate marker for atherosclerosis, exposes its limitations, arguing that this was probably due, at least partly, to the relatively poor data about IMT progression (Adams, Nakagomi et al. 1995, Bots, Baldassarre et al. 2007). More recently, Den Ruijter *et al.* (Den Ruijter, Peters et al. 2012) have published a meta-analysis including 14 population-based cohorts (45 828 patients) for a median follow-up of 11-years. Classical Framingham risk scoring was performed together with the extended model, which included common carotid IMT measurements to estimate the absolute 10-year risk for developing first-time MI or stroke. The net reclassification improvement with the addition of IMT was small (0.8%; 95% CI, 0.1-1.6%). In those at intermediate risk the net reclassification improvement was 3.6% in all individuals (95% CI, 2.7-4.6%). The conclusion was that the addition of IMT to the traditional Framingham risk score offered little improvement to the 10-year prediction of first-time MI or stroke, with unlikely clinical benefits. However, all these studies were done in the general population for which the Framingham score was devised. It could be argued that in SLE, the impact of re-stratification using the IMT could potentially be greater as the CVD risk in these patients is notoriously difficult to estimate through standard methods.

Notwithstanding this, further studies (Rundek, Arif et al. 2008) appear to suggest that the use of plaque area and texture may offer a stronger correlation with CVD-related events as will be discussed below.

1.5.2.2: 2D Plaque analysis

B-mode US of the carotid artery permits direct visualization of the systemic arterial vasculature. Furthermore, it allows the measurement of the thickness of the arterial wall as well as the detection of plaque, its location, size and nature each of which has a strong positive correlation with the occurrence of CVD-related events.

The definition of plaque is not homogeneous in the literature. According to the Mannheim Carotid IMT Consensus, plaque is defined as *“a focal structure that encroaches into the arterial lumen of at least 0.5 mm or 50% of the surrounding IMT value or demonstrates a thickness >1.5 mm as measured from the media-adventitia interface to the intima-lumen interface”* (Touboul, Hennerici et al. 2004, Touboul, Hennerici et al. 2007). In my own vascular US assessment, I have used a slightly different definition of plaque according to the standard practice of Professor Andrew Nicolaides and Dr Maura Griffin at the unit where the scans were carried out, as cited in their previous publications and epidemiological studies. Thus, for the purposes of this thesis, plaque was defined as *“a focal structure that encroaches into the arterial lumen of at least 0.5 mm or 50% of the surrounding IMT value or demonstrates a thickness >1.2 mm as measured from the media-adventitia interface to the intima-lumen interface”* (Ebrahim, Papacosta et al. 1999, Griffin, Nicolaides et al. 2002).

The risk factors for IMT thickening and presence of plaque appear to differ slightly: carotid plaques appear to be more influenced by conventional risk factors such as age, gender, type 2 diabetes and smoking (Moskau, Golla et al. 2005).

Carotid plaque is more frequently observed in the bulb region compared to the common carotid artery. Two explanations can be offered to justify this difference: on the one hand, the blood flow in the bulb area is turbulent, as opposed to the laminar blood flow typically observed in the common carotid, and this increases shear force stress on the arterial wall, facilitating endothelial injury in the bulb. On the other hand, even in the presence of plaque, the medial layer in the bulb area is much thinner than that of the common carotid where it is more common to find IMT thickening and plaque concomitantly. This suggests that a purely atherosclerotic process is involved in carotid plaque formation in the bulb region, while a combination of atherosclerosis and arteriosclerosis could be present in common carotid plaque formation (Li, Duncan et al. 1994).

The presence of carotid plaques has been shown to be an independent risk factor for cardiovascular events, with a much higher positive predictive value than IMT thickness (Griffin, Nicolaides et al. 2002). There is a difference between CCA IMT versus bulb IMT and plaque: the first correlates better with cerebrovascular disease while the latter are more directly associated with coronary artery disease-related events (Allan, Mowbray et al. 1997). Although the presence of thickened IMT in the CCA may be a precursor for plaque formation it is not a necessary condition, as epidemiological studies show that there can be plaque in the carotid bulb in the absence of increased CCA IMT thickening (Bonithon-Kopp, Touboul et al. 1996, Prati, Vanuzzo et al. 2006).

The relevance of the positive predictive value of carotid plaque (in either CCA or bulb) for coronary artery disease-related events (MI and death) was firmly established through a series of large-scale population studies. The most relevant are summarized in Table 3.

Study	n (% women)	Age (years)	Follow- up (years)	Carotid US parameters	Endpoint	HR for this endpoint in patients with plaque (95% CI) ^a
Tromsø study (Johnsen , Mathiesen et al. 2007)	6,226 (52)	25-84	6	Plaque area at CCA, bulb and ICA	MI	3.95 (women) 1.56 (men)
APSIS (Held, Hjemdahl et al. 2001)	558 (33)	60 (SD 7)	3	Plaque at CCA, bulb, ICA, ECA and CFA	MI/ CAD death	1.83-2.00
KIHD (Salonen and Salonen 1991)	1,228 (0)	42-60	2.5	n plaque vs. non-stenotic plaque vs. stenotic plaque	MI	Non-stenotic: 4.15 vs. Stenotic: 6.71
ARIC (Hunt, Evans et al. 2001)	12,375 (54)	45-64	5.2	Plaque at CCA, bulb and ICA	MI/ CAD death	1.04-1.78
Rotterda m study (van der Meer, Bots et al. 2004)	6,389 (62)	≥55	7-10	Plaque at CCA, bulb and ICA	MI	1.83

Table 3: Carotid plaque as a prognostic indicator for acute coronary events (adapted with permission from (Nicolaidis 2011))(n: number subjects; HR hazard ratio; CI confidence interval; APSIS: angina prognosis study in Stockholm; ARIC: atherosclerosis risk in communities study; KIHD: Kuopio ischaemic heart disease study. ^a adjusted for age, gender and conventional risk factors).

Overall, all these studies suggest that, regardless of the location, the presence of carotid plaque is associated with an increased risk for ACS. Furthermore, it is suggested that greater plaque area and the presence of >50% stenosis can be associated with further increase in the risk for ACS. Recently, Johri et al showed that in patients with carotid plaque (IMT ≥ 0.14 cm) there was significant increase in moderate (luminal narrowing greater than 50% of at least one segment or severe coronary artery disease) or severe (luminal narrowing of at least 70% within any segment of the main artery branches) coronary artery disease compared with those with no carotid plaque (IMT < 0.14cm) (46 vs 3 $p < 0.001$) (Johri, Chitty et al. 2013).

Besides quantitative plaque characterization, i.e. thickness and area, the plaque composition has also been shown to correlate with the risk of CVD-related events. As described above, an atherosclerotic plaque is made of varying proportions of lipids, smooth muscle cells, fibrotic tissue, platelets and inflammatory cells such as macrophages and neutrophils. Due to the different way each of these components interacts with the sound waves used in US, the aspect of a plaque in US is greatly influenced by its composition. Whilst lipid deposits are echolucent (black), calcium deposits and connective tissue are echogenic (white) and therefore, depending on the relative amount of these components, the US image of the plaque can offer an idea of the plaque composition. Calcified and non-calcified material can be distinguished easily as sound waves cannot travel through calcified deposits. This means that when an echogenic area is made of calcified material, there is a posterior shadow. In contrast, non-calcified echogenic areas allow for US to penetrate and be reflected back thus not eliciting the same shadowing effect. Initially it was thought that these non-calcified echogenic areas were composed of fibrotic tissue. However, studies using contrast US using micro bubbles have shown that they are actually rich in small blood vessels which correspond histologically to neo-vascularization within the plaque. These hyperperfused areas are thought to correlate with the presence of macrophages (Shah, Balan et al. 2007, Griffin, Kyriacou et al. 2010).

Based on these principles, a scale ranging from 1-5 has been devised to characterize different types of plaque according to their visual aspect (Figure 7). A type 1 plaque is almost exclusively echolucent i.e. >80% of the plaque appears to be black in US (Figure 7-A and 7-Aa). A type 2 plaque is predominantly echolucent (>50% black) but some non-echolucent components start to appear (Figure 7-B). A type 3 plaque is constituted by echolucent and echogenic material in similar proportions (Figure 7-C) while a type 4 plaque is almost exclusively echogenic but has no posterior shadowing as its echogenicity is given by the presence of collagenous or fibrotic tissue (Figure 7-D and 7-Dd). Finally, a type 5 plaque is

composed predominantly calcified material, hence a posterior shadowing effect is observed obscuring the image (Figure 7-E and 7-Ee).

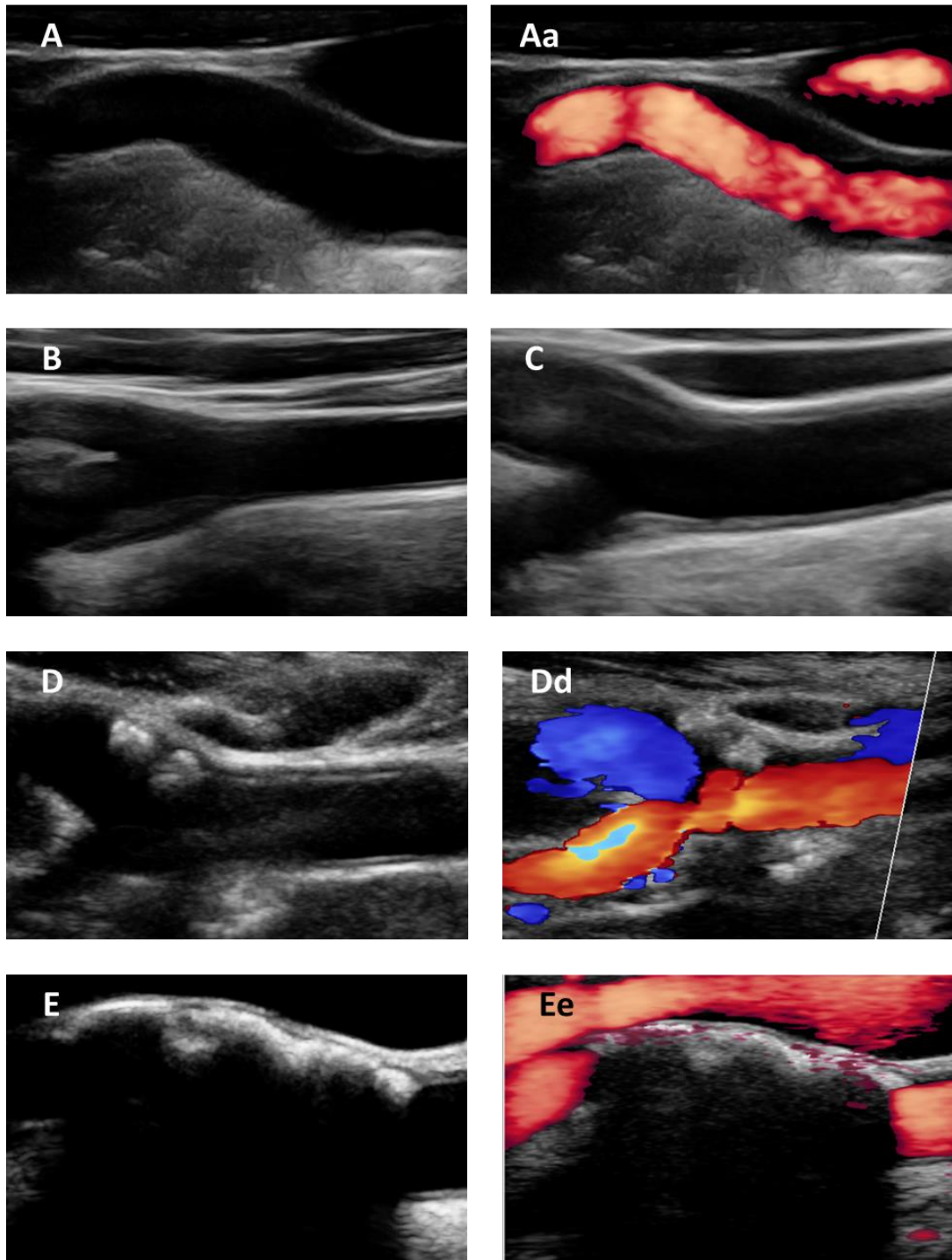


Figure 7: Qualitative score for plaque echostructure based on its US appearance (relative predominance of echolucent and echogenic components). **Type 1 (A and Aa):** >80% echolucent structure; **Type 2 (B):** >50% echolucent but echogenic components start to become apparent; **Type 3 (C):** similar proportion of echogenic and echolucent elements; **Type 4 (D and Dd):** predominantly echogenic but no posterior shadowing is observed; **Type 5 (E and Ee):** calcified plaque with posterior shadowing obscuring the lumen and far wall).

The type of plaque has been shown to correlate with the risk of stroke in a large scale observational study, the “Asymptomatic Carotid Stenosis and Risk of Stroke (ACSRS) study” (Nicolaidis 1995, Nicolaides, Sabetai et al. 2003). This study followed up 1115 subjects with asymptomatic carotid plaque during a mean 84 month period and found that 94% of strokes had happened in patients who had type 1, 2 or 3 plaques. Furthermore, when comparing the relative risk of stroke, plaques 1-3 had a 5 fold increased risk of being associated with stroke compared to type 4 and 5 plaques. Moreover, even when comparing type 1 or 2 plaques with type 3 or 4, the first were associated with twice the risk of stroke. This finding suggests that the type of plaque may be as important as its size in terms of risk of stroke.

The main limitation to this classification method is that is observer-dependent and therefore there is a degree of variability which can be particularly relevant in plaques with borderline appearance. As previously discussed, the use of normalization can minimize variability by standardizing resolution, equipment-related variables and image acquisition differences. The use of automated software which is able to determine objectively the texture features of plaques further minimizes variability. US images are coded in a grey-scale ranging from black to white which enables the software to recognize different patterns or textures. The underlying basis for texture analysis software is the Grey-scale median (GSM). The basis for this colour coding system is Haralick’s texture features (*Textural Features for Image Classification*, 1973) which relies on the principle of grey-level co-occurrence matrix. This value is obtained through a series of complex equations which compare the number of grey pixels in an image with the blood (black) and adventitia (white) as reference points (Tesar, Shimizu et al. 2008).

Therefore, with plaque texture analysis software, the GSM is calculated as a numerical value, with lower values corresponding to more echolucent i.e. lipid-rich plaques thus eliminating the subjective component of the previously described classification method. However, qualitative plaque texture classification is still useful when texture analysis software is not available, provided that it is done by an experienced observer and that inter-observer variability is low.

The use of texture analysis software also allow for further plaque composition characterization. It identifies the presence of black areas near the lumen and determines their area. This corresponds to the necrotic core previously described, which is associated with a greater risk of plaque rupture, particularly if a thin fibrous cap is present. The software also identifies discrete white areas within the plaque which correspond to the presence of neo-vessels.

In my thesis I will use both the qualitative visual scale plaque classification and the GSM calculated by image analysis software as specified in the Material and Methods section.

1.5.2.3: 3D Plaque analysis

3D imaging relies on the principle of image reconstruction using serial 2D pictures and can be applied to a range of methods like CT, US or MRI. The use of 3D US was first developed by Fenster *et al.* in the context of prostate cancer (Tong, Downey *et al.* 1996, Fenster, Downey *et al.* 2001). Its potential use in vascular imaging particular in carotid arteries was explored with a view to assess carotid atherosclerotic burden (Landry, Spence *et al.* 2004). The 3D US technique relies mainly on the type of probe used as well in specialized software that can then reconstruct the final 3D image from a series of consecutive 2D “slices”. The precise methods through which this is done are extremely complex and outside the scope of this thesis and therefore I will not explore them. Grossly, the US probe used in 3D US has a particular arrangement of the piezoelectric crystals that generate and receive the echoes which compose the US image (Figure 8).

The use of 3D US acquisition methods has evolved from a free-hand technique that required the translation of a transducer over the artery while 2D images were acquired to form a volumetric dataset to the use of automated single sweep 3D imaging methods, allowing for the acquisition from a stable probe position (Johri, Chitty *et al.* 2013, Kalashyan, Saqqur *et al.* 2013).

These measures allows the transducer to code the visual information obtained as a matrix of points (“voxels”) representative of the volume scanned which are then processed by specialized software ultimately producing a 3D image (Chiu, Egger *et al.* 2006, Egger, Spence *et al.* 2007). After image processing by the software, the end result is an initial image which corresponds to a conventional one obtained by 2D US (acquired phase) and 3 further images which would correspond to the constructed images produced by the software in different perspectives (i.e., if the acquired phase was obtained in a transverse cut, the constructed images would be longitudinal and coronal views) as well as a final 3 D reconstruction.

An example of this is shown in Figure 9 where a grape is depicted. Further explanation of the method and software will be offered in the methods section.

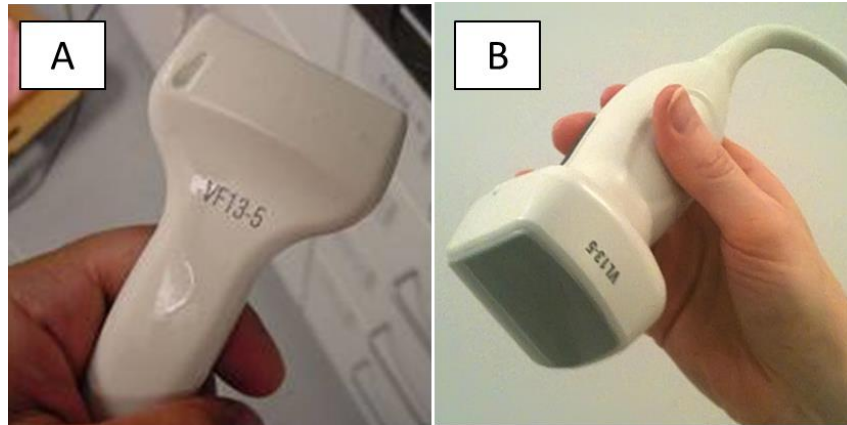


Figure 8: Vascular US probes: Conventional linear vascular US probe (A) and 3D US probe (B).

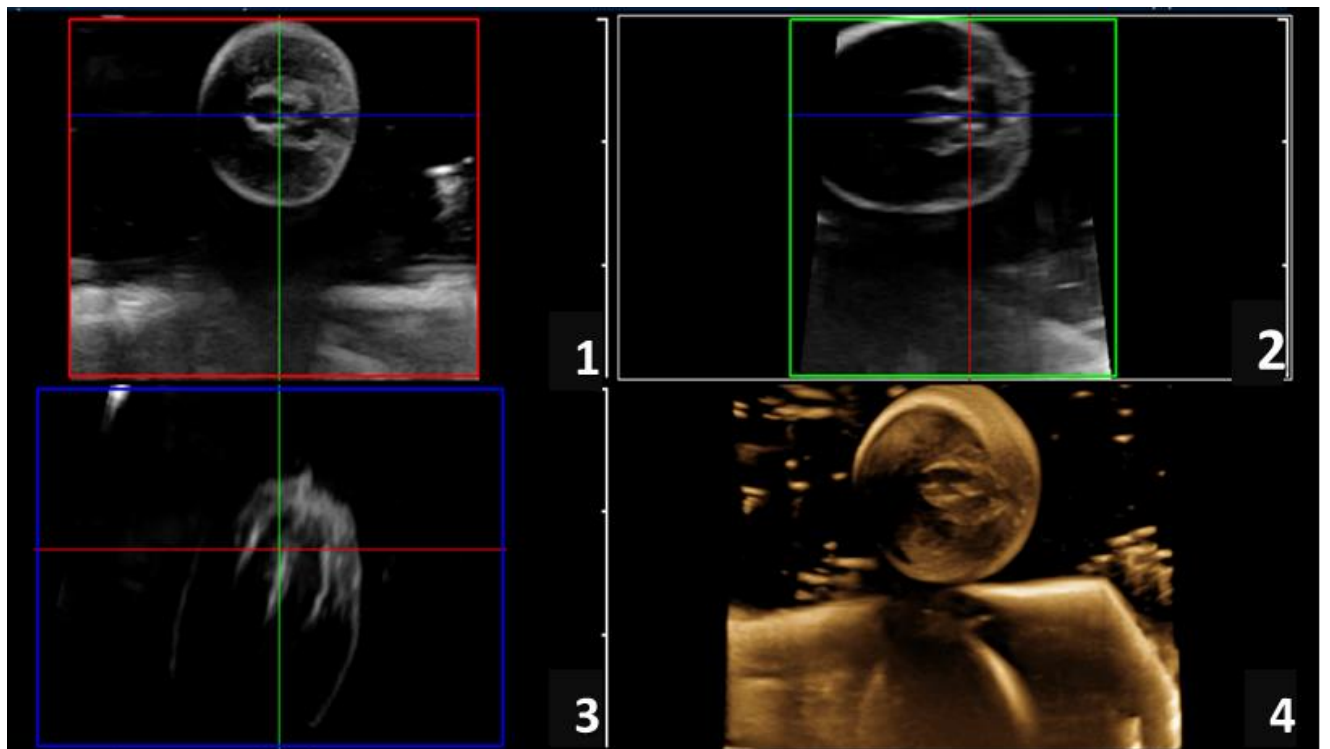


Figure 9: 3D US of a grape: 1 represents the acquired phase (transverse view). 2 and 3 are constructed views (coronal and longitudinal respectively). 4 is the 3D reconstructed image based on the acquired and constructed views.

One of the great advances made by 3D US was in vulnerable plaque identification and assessment. It is much more sensitive than conventional 2D US techniques in detecting small plaque ulcerations and it has a higher resolution which allows it to detect fibrous cap thinner than 200µm which is below the detection limit of most conventional US probes. Moreover, 3D imaging allows for accurate measurement of the volume of juxtaluminal black areas which are known to represent a major risk factor for plaque rupture. The accuracy of 3D US parallels that of intra-vascular US with virtual histology which has shown to have overall predictive accuracies greater than 93% in discriminating plaque components, i.e. fibrotic tissue, fibrofatty tissue, necrotic core and calcium (Diethrich, Paulina Margolis et al. 2007, Nair, Margolis et al. 2007).

Similar to what has been previously described for the GSM in 2D plaque imaging, a mean grey value (MGV) can be obtained in 3D US. Heliopoulos *et al.* performed 3D US in 110 symptomatic (stroke) and 104 asymptomatic atherosclerotic plaques. The plaques' MGV was determined and it was found that symptomatic patients had significantly lower MGV than asymptomatic patients when considering plaques producing less than 70% stenosis (Heliopoulos, Vadikolias et al. 2008).

In addition to texture features, 3D US can also determine the volume of both vessel wall and plaque. The relation between these two variables is much more accurate in assessing the evolution of plaque size over time than conventional 2D US. In the clinical setting, 3D US can be a sensitive way to assess the response to treatment with lipid lowering agents at plaque level. Ainsworth et al. reported on the effect of atorvastatin on plaque volume and were able to detect significant changes after only three months of treatment (Ainsworth, Blake et al. 2005). Thus, 3D US could provide alternative intermediate end points to assess the efficacy of treatment approaches in CVD.

Johri et al compared the predictive value of 2D and 3D carotid ultrasound for the presence of coronary artery disease (CAD) and found that the presence of plaque identified by either of these two methods had a high positive predictive value for the presence of CAD. Using an IMT-thickness cut-off of 0.14cm, the 2D carotid US yielded 93.9% sensitivity while a threshold of 0.09mL of plaque volume showed 98% sensitivity for the presence of CAD. However, the negative predictive value of 3D US was significantly higher than that of the 2D assessment, with a specificity of 93.3% vs 75% respectively (Johri, Chitty et al. 2013).

Makris et al recently reviewed the clinical evidence regarding the reproducibility and effectiveness of 3D ultrasound in carotid plaque characterization and concluded that besides good reproducibility in the assessment of various plaque features, it appears to yield

a an increased sensitivity to identify plaque changes post-treatment (Makris, Lavidia et al. 2011). Nonetheless, there are still no solid data to confirm the superiority of 3D US compared with the traditional 2D approach. Further research to establish the cost-effectiveness and increased sensitivity in identifying vulnerable plaques and/or re-stratify patients with asymptomatic carotid stenosis is required.

On balance, automated 3D carotid US is a reproducible, non-invasive method which can assess the presence and characterize atherosclerotic plaques in a way which appears to be as reliable as the traditional 2D ultrasound (Kalashyan, Shuaib et al. 2014) with at least similar sensitivity and specificity for CVD-risk prediction.

1.5.3: Vascular US use for assessing cardiovascular disease in the context of SLE

In a recently published review, the current options for imaging assessment of CVD in SLE were explored (Croca and Rahman 2012). The roles for MRI and CT based imaging techniques for evaluating SLE-associated myocardial dysfunction and both coronary and peripheral (carotid) artery atherosclerosis have now been largely explored. In some instances, such as cardiac magnetic resonance for assessing subclinical myocardial dysfunction, the sensitivity is undisputed but its cost and real-life clinical benefits prevent its more generalized use (Been, Thomson et al. 1988, Mavrogeni, Spargias et al. 2009, O'Neill, Woldman et al. 2009). Other methods are considered unsuited for assessing asymptomatic patients due to either radiation exposure or procedure risk deriving from their invasive nature.

Carotid US is an accurate, non-invasive method for assessing atherosclerotic disease through the measurement of the IMT and plaque quantitative and qualitative characterization. The prevalence of carotid plaque in the context of SLE ranges from 17% to 40% (Manzi, Selzer et al. 1999, Svenungsson, Jensen-Urstad et al. 2001, Doria, Shoenfeld et al. 2003, Roman, Shanker et al. 2003, Selzer, Sutton-Tyrrell et al. 2004, Wolak, Todosoui et al. 2004) whereas in control groups of healthy women its mean prevalence is 18% (Svenungsson, Jensen-Urstad et al. 2001, Roman, Shanker et al. 2003, Thompson, Sutton-Tyrrell et al. 2008). The Aging Vascular Study (EVA) which assessed the presence of carotid plaque in a cohort of asymptomatic women between the ages of 59 and 71 years show a prevalence of plaque of 19% which is still lower than the average 30% reported for much younger SLE-cohorts (Zureik, Ducimetiere et al. 2000).

Manzi *et al.* (Manzi, Selzer *et al.* 1999) reported on the use of B-mode carotid US in a cohort of 175 patients with SLE, 15% of whom had a previous CVD-related event. IMT and number of plaques and plaque thickness were considered. It was reported that 40% had at least one focal plaque and over 20% had at least one plaque occupying >50% arterial lumen. A higher prevalence of plaque was found in patients with higher cumulative damage measured by the modified SLICC damage score and with higher cumulative steroid dose and duration of treatment. The same group reported on the longitudinal progression of plaque size and IMT thickness comparing a cohort of 217 patients with SLE and 104 matched healthy controls (mean follow-up period of 5 years) (Thompson, Sutton-Tyrrell *et al.* 2008). They reported progression of plaque in 27% of patients (defined as an increase in plaque thickness and/or number of plaques) and a mean rate of IMT increase of 0.011mm/year. Plaque progression was significantly higher in the lupus cohort compared to healthy controls (27% vs. 10%) but no differences were found in IMT progression, though this may have been different if a longer follow-up period had been observed. The same group revisited the assessment of B-mode imaging of carotid arteries in a cohort of 392 women with SLE and no previous CV events (Kao, Lertratanakul *et al.* 2013). In this prospective, observational study including 392 adult women with SLE (mean age was 43.5 years) under follow-up for at least 8 years, 38 patients had a cardiovascular event and 17 had suffered what was defined as “hard event” including myocardial infarction, fatal cardiac arrest and cerebrovascular accident. Increased baseline IMT and presence of plaque was predictive of all types of cardiovascular events (HR 1.35 and 4.26 respectively), independently of traditional CV risk factors and medication use. It was thus concluded that women with SLE without previous CV events, carotid IMT and plaque were predictive of future CV events.

The factors more strongly associated with increased IMT were increased serum creatinine and lower diastolic blood pressure. The presence of impaired kidney function closely correlates with the presence of vascular damage (O'Rourke and Safar 2005). Therefore it can be hypothesized that systemic inflammation associated with SLE is involved in vascular damage which is relevant for the pathophysiologic processes underlying both CVD and kidney dysfunction (Doria, Shoenfeld *et al.* 2003, Manger, Kusus *et al.* 2003). Low diastolic blood pressure on the other hand, is a well-known marker of arterial stiffening which in turn is considered to be the precursor of atherosclerosis as it marks the presence of endothelial dysfunction (O'Rourke, Staessen *et al.* 2002). With regards to plaque progression, it was found that the most influential factors were higher triglycerides and C3 levels as well as the use of immunosuppressants at baseline. Although the link between higher triglycerides and plaque progression is not surprising, the same cannot be said for the relation with higher C3 levels and immunosuppressant use. Higher C3 levels have been previously described in

association with coronary calcifications (Manger, Kusus et al. 2003) but the links between complement levels and vascular disease are yet to be clarified.

To my knowledge, no studies have been done using plaque area or texture feature analysis in SLE nor have there been any reports on the use of 3D US in this specific population.

In summary, the use of carotid US in the assessment of atherosclerosis in the context of SLE has already been explored but its full potential, particularly in asymptomatic patients, is yet to be achieved. Ideally, a link between disease-related markers, either traditional (C3, anti-dsDNA) or novel (nitration measures, anti-apoA1) would be established. Thus, when a patient was diagnosed with SLE and presented a serological profile pointing towards a higher CVD-risk, carotid US imaging could be used to assess her baseline atherosclerotic profile and follow-up its evolution over time. The combination of clinical assessment, serological profiling and imaging studies could prove to be the key for early diagnosis and primary prevention of CVD related events, and thus allow for mortality and morbidity reduction among patients with SLE.

My aim in this part of my thesis is to assess the presence of plaque in patients with SLE who are completely asymptomatic for CVD, i.e. they have not had strokes/ transient ischaemic accidents (TIA), MI/ angina and peripheral vascular disease/ intermittent claudication. Furthermore, I aim to establish the prevalence of plaque and characterize it in terms of its composition/ texture, area/ volume and correlate these data with the patients' characteristics.

2: AIMS

Stemming from the concept of increased atherosclerotic burden among patients with SLE and bearing in mind the theoretical background established in the Introduction, one of my key goals in this thesis is to assess the presence of plaque in a cohort of 100 patients with SLE with no prior history of cardiovascular disease –related events or symptoms suggesting that underlying, clinically significant atherosclerosis is present by performing high-sensitivity vascular ultrasound scans of the carotid and femoral territories.

Besides obtaining IMT measurements and establishing plaque prevalence, I aim to fully characterize each individual plaque in terms of its composition/ texture as well as its area/ volume. Additionally, I will compare the data from the SLE cohort with a group of age and sex matched non-SLE controls that underwent similar ultrasound assessment and establish whether there are differences in IMT thickness, plaque prevalence, size and texture.

The vascular ultrasound cohort will be extensively characterized including demographics, SLE-associated features (organ involvement, disease activity indexes and classic auto-antibody profile), general metabolic profile (including lipid profile, renal function and blood pressure measurements), treatment and specific, not standardly used serological tests. In this cross-sectionally cohort of patients I have chosen to assess antibodies directed against both HDL and anti-apoA1 (IgG and IgM subclasses) as well as to determine levels of nitrated nucleosomes and nitrated apoA1 as they could be a surrogate marker for endothelial activation.

Aiming to comprehensively characterize protein nitration (nucleosomes and albumin) and anti-apoA1 IgG antibodies levels over time, I have assessed these two targets in a separate, longitudinal cohort of patients, using multiple samples collected over time and spanning a representative period of each individual patient's disease course (periods of flare and remission) . This will allow me to establish if these two variables vary over time and whether they correlated with treatment, auto-antibody profile and disease activity.

Additionally, I aim to determine the prevalence of anti-apoA1 antibodies early in the course of SLE and whether this influences patient outcome, namely in terms of mortality and morbidity, particularly in what concerns cardiovascular events.

After carrying out these tasks I will be able to establish the prevalence of asymptomatic atherosclerosis in a cohort of patients with SLE with no clinically evidence of cardiovascular disease and determine IMT, plaque distribution, size and echogenicity/ texture. Moreover, I will be able to report on the impact of non-SLE related variables (lipid profile and blood

pressure) and SLE-related factors (disease activity and auto-antibody profile) on IMT and plaque characteristics.

The overarching hypothesis throughout this thesis is that patients with SLE have subclinical atherosclerosis and that the presence of increased endothelial activation indirectly assessed by the measurement of nitrated proteins, the presence of increased inflammation due to greater disease activity as well as specific auto-antibodies, particularly those directed against the HDL complex may be associated with increased likelihood of plaque/ IMT thickening. If this hypothesis is confirmed, it would make a strong argument for the screening of all patients with SLE to allow for early recognition of those who may have increased cardiovascular risk and would thus benefit from closer monitoring and early treatment in order to prevent mortality and morbidity.

3: MATERIALS AND METHODS

3.1: Patient selection

Throughout my research project, I have assessed three distinct, but overlapping, groups of patients with SLE: a longitudinal cohort comprised of 397 samples taken from 49 patients (longitudinal group); a cross-sectional cohort of 499 samples taken from 499 individual patients within two years of SLE diagnosis (early disease group) and a cross-sectional cohort of 100 patients with SLE with no history or symptoms suggesting CVD selected to undergo vascular US (vascular US group). Figure 10 represents the three cohorts considered and the number of patients overlapping between them.

For my serological studies, I selected 100 healthy controls and 17 patients with rheumatoid arthritis (RA). All the patients with SLE fulfilled the revised ACR classification criteria. Comprehensive demographic and clinical characterization was obtained for all the patients with SLE, including auto-antibody profile, disease activity profile and treatment, namely steroid dose at the time of sample collection as well as immunosuppressant regimen when applied (mycophenolate mophetil, azathioprine, cyclophosphamide, cyclosporine and B-cell depletion) and whether the patient was taking hydroxychloroquine.

I was able to access vascular US data from a non-SLE cohort in Cyprus, who had been scanned using the same protocol that I used. Though these scans were not done by me and I had no involvement in acquisition of the data, I have used them as a control cohort for comparison with the vascular US data that I obtained in patients with SLE. For this reason I have described this non-SLE cohort below.

I will now offer a more detailed description of each of these cohorts and their selection criteria.

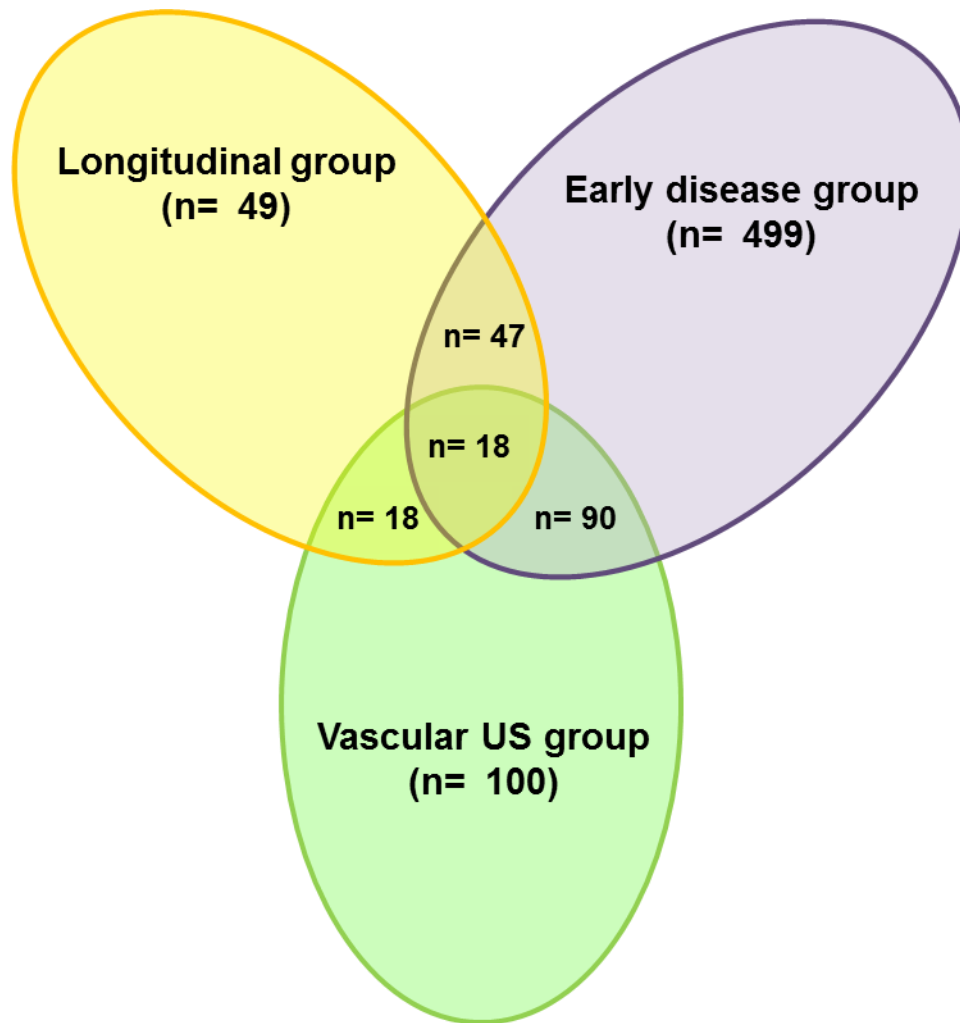


Figure 10: Venn diagram depicting the three overlapping cohorts assessed. The longitudinal cohort included 49 patients of which 47 were present on the early disease cohort and 18 on the vascular ultrasound group. The early disease group was comprised of 499 patients, 90 of which were also included on the vascular ultrasound cohort. Eighteen patients belong to all 3 groups.

Longitudinal group

Longitudinal serum samples ($n= 397$) were selected retrospectively from a cohort of 49 patients with SLE. On average, each patient had 8 samples (SD 2.16; min 3; max 14) covering a mean follow-up of 89 months (SD 46; min 14; max 180). Patients who had suffered flares of disease activity in different organ systems during the follow-up time considered were preferentially selected and examples of flares in all the main organs and systems were included.

For all samples where data were available (94% of samples), I obtained anti-dsDNA and complement C3 levels and disease activity from the date of the sample and from the previous three assessments. Anti-dsDNA and C3 were measured in the routine clinical laboratory at UCLH using enzyme-linked immunosorbent assay (ELISA) (Shield Diagnostics, Dundee, UK) and laser nephelometry respectively. Data on ethnicity, gender, drug therapy and anti-Sm, anti-RNP, anti-Ro and anti-La (all tested by ELISA) status of the patients were obtained from the clinical records of the patients.

For the longitudinal group, measurements of NN, NA, anti-NCS and anti-ApoA1 IgG levels using the assays described below were performed.

Early disease group

Since the University College Hospital (UCH) Lupus clinic began (in 1978) sera from all the patients followed up over the years has been routinely collected and stored at -80°C for the purposes of research, following informed consent according to ethical approval (described below). From this vast cohort of stored sera samples, 499 samples obtained from 499 patients within two years of diagnosis were selected. The earliest sample was obtained in 1978 and the last in 2011. All patients had been under continuous follow-up between one and 34 years (mean 12.1 years). 47 of these patients were also in the longitudinal group but different samples from later time-points in the disease course were used.

Data on age at diagnosis, ethnicity, death, CVD-related events and auto-antibody profile were also obtained in >90% of samples. Data on SLICC damage scores were only available for 236 patients who had been previously included in a retrospective study assessing the 10 year-follow up data (Chambers, Allen et al. 2009).

In the early disease group, only anti-ApoA1 IgG levels were measured using the ELISA protocol described below.

Vascular ultrasound group

100 patients with SLE and with no history of CVD-related events or symptoms suggestive of underlying CVD, including angina, intermittent claudication and TIA were selected. Of these, 90 were also in the early disease group and 18 were in the longitudinal group. Vascular ultrasound scans of both carotid and femoral territories (common carotid, common femoral and their bifurcations) were carried out as described in section 3.4: "Vascular ultrasound of carotid and femoral arteries." Data on disease activity, ethnicity, auto-antibody profile and treatment were obtained from the patient's clinical records.

Sera belonging to the patients included in this group were tested for NN, nitrated ApoA1, total ApoA1, anti-ApoA1 antibodies (IgG and IgM) and anti-CCP antibodies (IgG and IgM).

The anti-CCP IgG and IgM levels were measured by Dr Geraldine Cambridge using an ELISA protocol previously published (Cambridge, Stohl et al. 2006).

Healthy controls – serological tests

I tested samples from 100 healthy control subjects, which were originally obtained as part of the Health Survey for England (HSE) 2006 and were used with the permission of the Health and Social Care Information Centre and NatCen Social Research (Copyright © 2008, all rights reserved). Anonymised data on age, gender and ethnicity of the subjects as well as confirmation of the absence of long term illness or history of CVD were also provided by the same organization.

Disease controls – serological tests

I selected 17 patients with RA and a small cohort of patients with myositis (n= 12) and Sjogren's syndrome (n= 13). These samples were obtained from patients under regular follow-up at the General Rheumatology Outpatient Clinic at UCLH and had previously given informed consent regarding sera storage and authorization for broad use in research carried out by the Centre for Rheumatology.

Non-SLE controls – vascular US

In order to have a control non-SLE cohort who had undergone assessment using a similar vascular US protocol to that which I have performed, the patients I selected were matched with patients from the Cyprus Epidemiological Study (Griffin, Nicolaides et al. 2009) . In this study, volunteers aged 40 years or older were recruited from three randomly selected towns in Cyprus. These individuals were selected regardless of the presence of known CVD or risk factors for CVD namely smoking, altered lipid profile or diabetes. Due to the age range of the

SLE patients, I could not find appropriate matched controls for patients less than 40 years of age. However, for 66 of the patients with SLE I had scanned, 2 age and sex matched non-SLE controls from the Cyprus study were selected. Unfortunately, it was not possible to match patients according to their ethnicity as the Cyprus population is mostly Caucasian origin. The scanning method I used was the same as that used to assess the Cyprus cohort, as well as the image analysis software. Furthermore, I was trained and had one-on-one supervision with Dr Maura Griffin who performed all the vascular US scans for the Cyprus study. Intra and inter-observer variability were determined to demonstrate reproducibility of the method. Thus, it is possible to compare our findings to those of the Cyprus group. The limitations of this method will be further explored in the Discussion section.

Ethical approval for all parts of the study was granted by the joint University College London/University College London Hospitals (UCL/UCLH) Research Ethics Committee (Reference 06/Q0505/79) and subjects gave informed consent for use of their stored serum samples. The 100 patients who underwent vascular ultrasound scanning gave separate informed consent for that procedure. The patients belonging to the Cyprus Epidemiological Study also gave separate informed consent before undergoing vascular US.

All the patients included fulfilled the revised ACR classification criteria for SLE (Hochberg 1997). The patients with SLE are/were under the care of the Lupus Clinic at UCLH and have been under continuous follow-up for between one and 34 years.

3.2: Definition of disease activity and laboratorial profile

Several serological variables were measured at the UCH associated clinical laboratory. ANA was measured by immunofluorescence with a Hep-2 substrate and a titre of $\geq 1:80$ was considered positive. Rheumatoid factor was measured by both latex testing and RA particle agglutination (RAPA) assay techniques (positive titre $\geq 1:80$). The ENA profile, including antibodies to Ro, La, Sm and RNP, were measured by ELISA. Anti-dsDNA was determined by ELISA (Shield Diagnostics, Dundee, UK) and immunofluorescence with *Crithidia luciliae* as the substrate. Anti-dsDNA positivity was considered if the *Crithidia* test was positive or if the ELISA result was at least twice the upper limit of normal (normal < 50 U/l) on two occasions. Complement component C3 was measured by laser nephelometry (normal 0.9g-1.8/L).

The classic BILAG was used to determine disease activity clinically (Hay, Bacon et al. 1993). Activity on the date of the sample (current activity) for each organ system was defined as high if the BILAG score was A or B and low if it was C, D or E. Disease activity was also

defined based on the numerical overall BILAG score: a score of less than 5 was considered to correspond to low disease activity while of score of 5 or above as high.

In order to reflect sustained disease activity, in the longitudinal group, the most recent four assessments were considered. Persistently low activity was defined as all systems BILAG C, D or E and persistently moderate-high disease activity was considered if there was either a score of at least one A or more than one B in any BILAG system on at least 2/4 occasions. Fewer than 10% of the samples did not fit into either of these categories and were therefore excluded from this analysis.

In the early disease group, data on damage at 5 and 10 years post diagnosis (and up to 20 years in some cases) had been obtained on 236 patients. These data were used as a clinical outcome measure in the assessment of this cohort. Damage was measured by the Systemic Lupus International Collaborative Clinics Damage Index (SLICC-DI).

For the patients included on the vascular US group, an extensive laboratory characterization was obtained from the UCH associated laboratory, including ESR, CRP, serum immunoglobulins (IgG, IgA and IgM), serum creatinine (creat), serum urea (urea), urine protein:creatinine ration (Up:C), albumin (alb), vitamin D (vitD), t-cholesterol, HDL, LDL, total cholesterol/HDL ratio (tC:HDL) and homocysteine. In this group, an exact match between date of scan (DOS) and date of assessment was not possible for all the variables. When not available at the DOS, the closest test taken within a year of the DOS was considered (>90% patients). When no tests had been taken within a year of the DOS I considered that data for that variable were unavailable.

Finally, for this group, besides getting the overall BILAG score at the DOS, full data on disease activity based on the BILAG score were obtained. For 80% of patients, these records covered all the follow up time at UCH since the diagnosis of SLE. For the remaining 20%, the mean disease period for which there was no record of BILAG score was ~8 years. Nonetheless, I included their data in the analysis but it must be stressed that data are not complete for the whole duration of the disease but for the duration of disease from the beginning of BILAG score recording. Based on these records, I considered different categories which attempt to portray disease activity over time for each patient:

- 1) Number of visits with ≥ 2 B flares and ≥ 1 A flare;
- 2) Mean monthly global BILAG score
- 3) Cumulative global BILAG score during the first year of follow up (sum of the global BILAG scores registered at every hospital visit during the first year after diagnosis);

- 4) Mean global BILAG score (sum of global BILAG scores divided by the number of visits).

These are not classical measures of disease activity, but they represent an effort to illustrate the way disease developed over time as patients with more active disease had not only higher global BILAG scores but also more frequent visits to hospital corresponding to periods of disease flare.

Data on sustained disease activity (i.e. persistently active or inactive) as described for the longitudinal group were also recorded.

3.3: Serological tests

The specific serologic tests carried out by myself where used differently on the different cohorts assessed, i.e. not all these assays were used in all the cohorts assessed. Table 4 summons which assays were performed in each individual cohort.

	Longitudinal cohort (n= 49)	Early SLE cohort (n= 499)	Vascular US cohort (n= 100)
Nitration:			
NN	☑	-	☑
NA	☑	-	-
NapoA1	-	-	-
Anti-NCS	☑	-	-
Total apoA1	-	-	☑
Anti-apoA1			
IgG	☑	☑	☑
IgM	-	-	☑
Anti-HDL IgG	-	-	☑
Anti-CCP			
IgG	-	-	☑
IgM	-	-	☑

Table 4: Different serological assays performed by the candidate for each individual patient cohort considered throughout this thesis.

With the exception of the measurement of anti-CCP IgG and IgM antibodies which was kindly undertaken by Dr Geraldine Cambridge as previously described, all the following ELISA protocols were developed, optimized and carried out by me. I tested the 499 samples of the early disease group for anti-ApoA1 IgG with the help of a BSc student, Maria Davari.

A few reagents and materials were common to the different ELISA protocols described below.

Some procedures were also shared by these ELISA protocols and include the covering of the plate with cling film during before each incubation period, except when the HRP substrate was added in which case the plate was covered with tin foil due to its light sensitivity.

For all the assays, net optical density (OD) was determined by subtracting the reading of the control side from the test side for each sample, to eliminate the contribution of non-specific background binding.

The alkaline phosphatase substrate (ALP) (KPL 50-80-00) was made up fresh for every assay by diluting one tablet of pNPP (nitrophenyl-phosphate) in a solution made up by adding 1mL of diethanolamine (DEA) buffer to 4mL of distilled water.

The OD values were converted into absorbance units (AU) by comparison to the standard curve obtained from the serial dilution of a positive control (PC) loaded onto every plate. The standard curve always included PC dilutions above and below the dilution selected to test the patients' sera. It was defined that the OD for the PC dilution corresponding to the dilution used for the sera samples was 100 AU. From the curve thus obtained, an equation was obtained and used to calculate the AU values for the individual serum samples tested. For each ELISA protocol, an example of the serial curve will be shown, including a curve equation and a R^2 value to demonstrate quality of adjustment between the curve and the trend line equation. This method was selected because, with the exception of the total apoA1 ELISA, the PC used was a pooled sera sample and a correspondence between OD and a known concentration could not be established.

When using the ALP substrate, 4 readings were taken at 15min intervals (15, 30, 45 and 60min). For all the assays using this substrate, the 60min reading was selected as it yielded the higher net OD.

All the protocols described were found to be reproducible with intra and inter-plate coefficient of variation of <10%.

3.3.1: Indirect ELISA for detection of nitrated nucleosomes

96-well Nunc-streptavidin plate (Thermo Scientific 236004) was divided in half: the test side was coated with biotinylated polyclonal goat anti-nitrotyrosine antibody (Abcam 27646) diluted 1:1000 in PBS (Gibco by Life Technologies 18912-014) and the control side coated with PBS (75µL per well). After one hour incubation at 37°C, the plate was washed 3 times with PBS-0.1% Tween (Sigma 274348). Blocking was done by adding 200µL per well of 0.5% ovalbumin (Sigma A2512) in PBS-0.1% Tween for one hour at 37°C. During this period, serum samples were thawed and diluted at 1:30 in PBS. A pooled sera sample was used as positive control (PC). After washing the plate 3 times with PBS-0.1% Tween, a 4-point dilution of the PC starting at 1:15 was loaded in duplicate on the first row of both the test and control half of the plate. Patients' serum samples (100µL/well) were added to both sides of the plate. After incubating for 1h at 37°C, the plates were emptied and washed 4 times with PBS-0.1% Tween. The primary antibody (rabbit anti-histone H3 antibody; sc-10809, Santa Cruz Biotechnology) was then diluted at 1:2000 in 0.5% ovalbumin in PBS-0.1% Tween and added to the plate (50µL per well). After 1h incubation at RT, the plate was washed 3 times with PBS-0.1% Tween. The secondary antibody (goat anti-rabbit IgG HRP conjugate; Sigma 0545) was diluted at 1:2000 in 0.5% ovalbumin in PBS-0.1% Tween and 50µL was added to each well. After incubating for one hour at RT and washing, HRP substrate (KPL 52-00-03) was added (100µL per well). Following a 10min incubation at RT, the reaction was stopped with TMB stop solution (KPL 50-85-06) and OD read at 450nm.

3.3.2: Indirect ELISA for detection of nitrated albumin

The ELISA protocol used to determine the NA levels was very similar to the one previously described for NN. The relevant differences are as follows:

- the blocking agent used was 0.5% agarose in PBS
- the primary antibody used was a rabbit polyclonal anti-human albumin antibody (Abcam 2406)

3.3.3: Direct ELISA for detection of anti-nucleosome antibodies

A 96-well Nunc-Maxisorb plate (Sigma M9410) was divided in 2 halves: the test side was coated with 50µL per well of nucleosome antigen diluted 1:500 in 20mM Tris/HCL buffer (pH 8.0) containing 0.15M of NaCl; while the control side was coated with the Tris buffer alone. The plates were incubated at 37°C for 2h and then emptied and washed 3 times with PBS-0.1% Tween. Blocking was done by adding 200 µL per well of 1% BSA-PBS-0.1% Tween

and allowing for 1h incubation at 37°C. During this period, the sera samples were thawed and diluted 1:50 in 1% bovine serum albumin (BSA) (Sigma A7030)-PBS-0.1% Tween. A pooled sera sample was used as a PC and a 4-point dilution starting at 1:25 was done in duplicate. After washing the plate, the sera samples (50µL/well) were loaded in duplicate into test and control side. The plate was left to incubate for 30min at 37°C and then washed 4 times with PBS-0.1% Tween. Afterwards, 50µL of rabbit polyclonal anti-human IgG HRP conjugate (sc-2778, Santa Cruz Biotechnology) diluted at 1:1000 in 1% BSA-PBST was added to each well. After incubating for 30min at 37°C, the plate was washed and HRP substrate was added (100µL per well). After 10min incubation at RT, the reaction was stopped with TMB stop solution and OD read at 450nm.

Figure 11 shows the typical standard curves for the NN (A), NA (B) and anti-NCS (C) ELISA protocols.

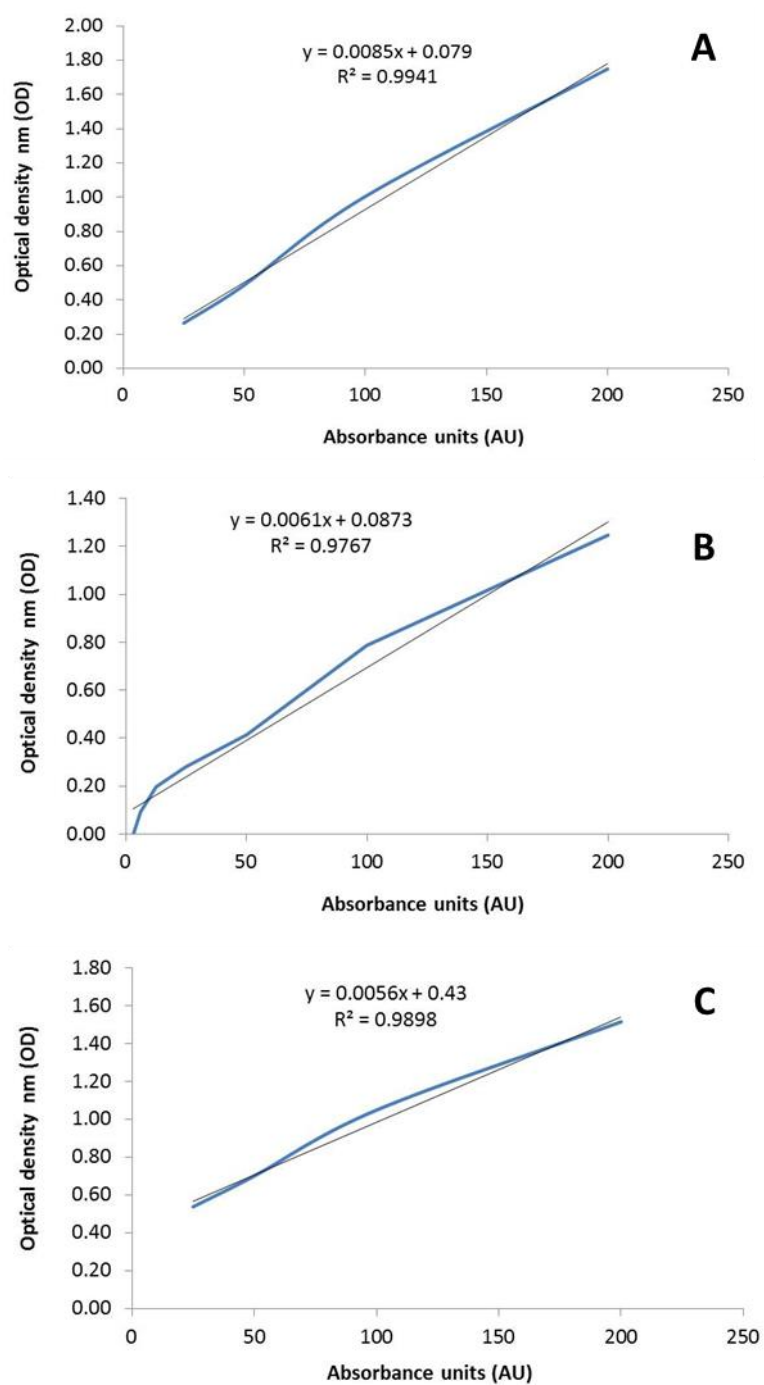


Figure 11: Typical standard curves for the NN (A), NA (B) and anti-NCS (C) ELISA protocols. A pooled sera sample was used as a PC for these assays. Absorbance units (AU) were determined based the curve equation obtained for each standard ($R^2 > 0.95$).

3.3.4: Direct ELISA for detection of anti-ApoA1 antibodies

3.3.4.1: Anti-ApoA1 IgG antibodies

A Nunc-Maxisorb 96 well ELISA plate was divided in half and the test side was coated with 50 μ L of 10 μ L/mL of ApoA1 (Sigma A0722) in 70% ethanol, whilst the control half of plate was coated with 50 μ L per well of 70% ethanol. The plate was incubated for 90min at 37°C and then emptied and washed 3 times with PBS. After tapping and removing all remaining washing buffer, the plate was blocked with 100 μ L per well of 1% BSA diluted in PBS and allowed to incubate for 1h at 37°C. During this time, the sera samples were thawed and diluted 1:50 in 1% BSA-PBS. After emptying and washing the plate 3 times with PBS, the sera samples were loaded unto the plate. All samples were tested in duplicate on both the control and test half. On the first row of each side of the plate, a 7-point dilution of the positive control was performed starting at 1:25 dilution on the first 2 wells and followed by serial dilutions down to 1:1600. The positive control used was pooled sera sample from 5 patients with SLE. After loading, the plate was incubated for 1 hour at 37°C and then emptied and washed 4 times with PBS-0.1% Tween. 50 μ L per well of goat anti-human IgG-alkaline phosphatase conjugate (Sigma 3150) diluted 1:1000 in 1% BSA-PBS was added and allowed to incubate at RT for 1h. Finally, 50 μ L of ALP substrate (KPL 50-80-00) was added to each well and absorbance read at 405nm at 15, 30, 45 and 60min.

3.3.4.2: Anti-ApoA1 IgM antibodies

A Nunc-Maxisorb 96-well ELISA plate was divided in half: the test side was coated with 50 μ L per well of 10 μ L/mL of ApoA1 in 70% ethanol and the control half with 50 μ L 70% ethanol. The plate was incubated for 90min at 37°C and then emptied and washed 3 times with PBS. After drying, the plate was blocked with 100 μ L per well of 1% BSA diluted in PBS and kept overnight at 4°C. The plate was again washed 3 times with PBS and allowed to dry. In the meantime, the sera samples were thawed, diluted 1:50 in 1% BSA and loaded unto the plate and tested in duplicate on both the control and test half. A pooled sera sample from 4 patients with SLE was used as the positive control and a 7-point dilution was loaded into first row of each side of the plate, as described above. After loading, the plate was incubated for 1 hour at 37°C and then emptied and washed 4 times with PBS-0.1% Tween. 50 μ L per well of goat anti-human IgM-HRP conjugate (Sigma A 6907) diluted 1:5000 in 1% BSA-PBS was added and allowed to incubate at RT for 1h. After washing 3 times with PBS-0.1% Tween, 50 μ L of HRP substrate was added to each well and allowed to incubate for 5min, when the reaction was stopped with TMB stop solution and OD read at 450nm.

3.3.5: Direct ELISA for the detection of anti-HDL antibodies

Human HDL (Sigma L1567) was diluted in distilled water to a concentration of 2mg/mL and stored as 1mL aliquots at -80°C. Smaller aliquots for everyday use were kept at -20°C. A Nunc-Polysorb 96-well ELISA plate (Sigma M0661) was divided into 2 halves: the test side was coated with 50µL per well of 20 µg/mL HDL in 70% EtOH while the control side was coated with 50uL per well of 70% EtOH. After incubating for 1h at 37°C, the plates were washed 2 times with PBS and allowed to dry. Blocking was done by adding 100µL per well of 1% BSA-PBS and leaving ON at 4°C. The plates were then washed twice with PBS and sera samples were loaded into the plate diluted 1:100 in 1% BSA-PBS (100µL/well). All samples were tested in duplicate. On the first row of either side of the plate, a 7-point dilution of the PC starting at 1:50 dilution was loaded. The PC was a pooled sera sample from 5 patients with SLE. The loaded plate was allowed to incubate for 1h at 37°C and then washed 3 times with PBS. The secondary antibody (anti-human IgG alkaline phosphatase - Sigma A3150) was diluted 1:1000 in 1% BSA-PBS and 50µL added to each well. Following 30min incubation at 37°C, the plate was washed 3 times with PBS and each well was loaded with 50 µL of ALP substrate. The plate was kept at 37°C and OD read at 405nm at 15, 30, 45 and 60min.

Figure 12 depicts the typical standard curves for the anti-ApoA1 IgG (A), IgM (B) and anti-HDL IgG (C) antibodies.

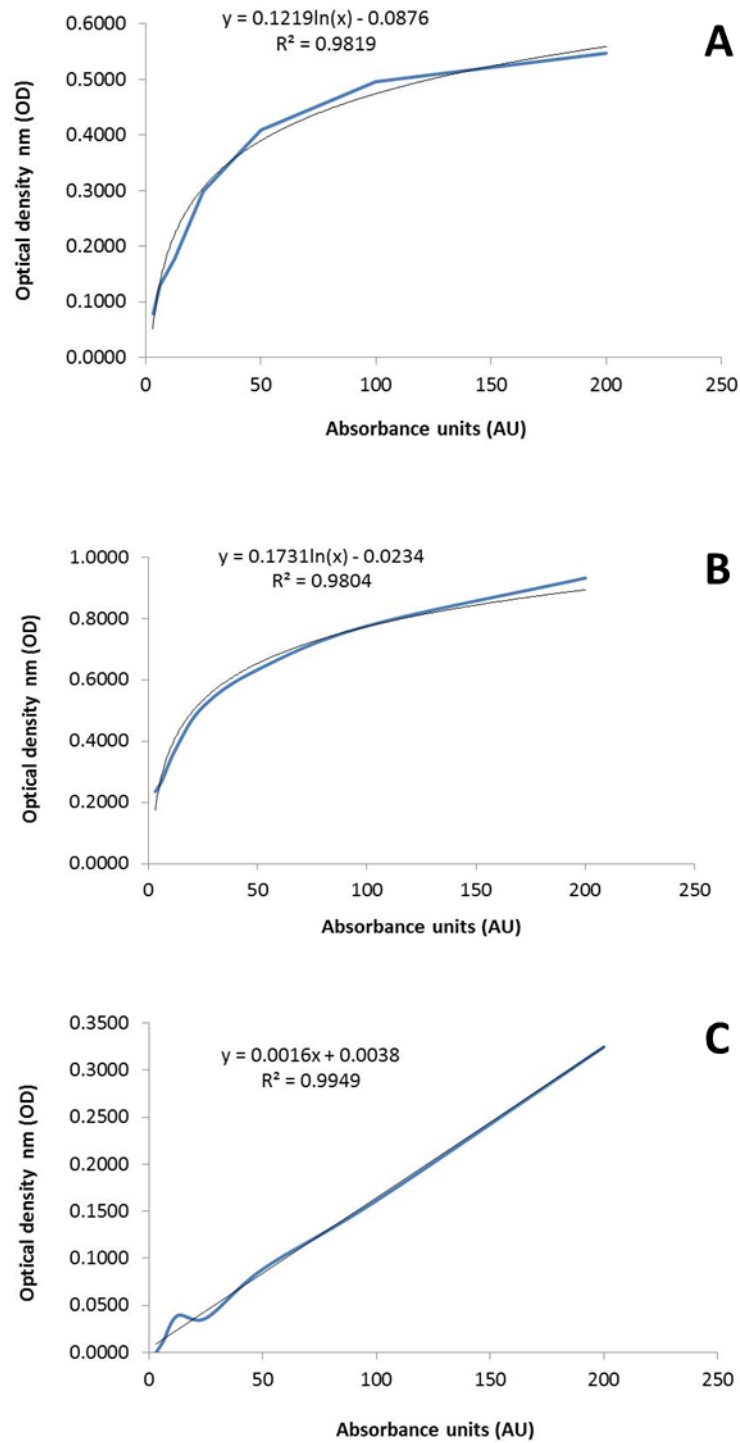


Figure 12: Typical standard curves for the anti-ApoA 1 IgG (A), anti-ApoA1 IgM (B) and anti-HDL IgG (C) ELISA protocols. A pooled sera sample was used as a PC for these assays. Absorbance units (AU) were determined based the curve equation obtained for each standard ($R^2 > 0.95$).

3.3.6: Indirect ELISA for the detection of nitrated ApoA1

A 96-well Polysorb plate was divided in two and the test side was coated with 75µL of goat anti-nitrotyrosine antibody (Abcam 27646) diluted 1:2000 in PBS while the control side was coated with PBS alone. After incubating for 1h at 37°C, the plates were washed 3 times with PBS and blocked with 150µL of 1%BSA-PBS per well. Following ON incubation at 4°C, the plates were washed 3 times with PBS and 50µL of each serum sample were added in duplicate to both test and control side (1:100 dilution in 1% BSA-PBS). A 7-point dilution of the PC was loaded on the first row on either side of the plate as previously described, starting at 1:50 dilution. The PC used was a pooled sera sample from 6 patients with SLE. The plates were allowed to incubate for 1h at 37°C, washed 3 times with PBS-0.1% Tween and 50µL of the primary antibody (mouse anti-human ApoA1 antibody - MAB011-A/13) diluted at 1:5000 in 1% BSA-PBS were loaded into each well. Following a 30min incubation at 37°C and 3 washes with PBS-0.1% Tween, 50 µL per well of rabbit anti-mouse IgG-HRP conjugate (Dako P0161) diluted 1:2000 in 1% BSA-PBS were loaded and the plate left to incubate for 30min at 37°C. During this incubation period, enough HRP substrate for the following step was kept at RT. The plates were then washed 3 times with PBS-0.1% Tween and 50µL per well of HRP substrate was added and allowed to incubate for 5min at RT when the reaction was stopped with 50µL per well of TMB stop solution. OD was read at 450nm.

3.3.7: Indirect ELISA for the detection of total ApoA1

A 96-well Polysorb plate was divided into test and control sides: the test side was coated with 50µL per well of mouse anti-human ApoA1 (MAB011-A/13) diluted 1µg/mL in PBS while the control side was coated with PBS alone. Following a 1h incubation at 37°C, the plate was washed twice with PBS, blocked with 150µL per well of 1% BSA-PBS and left ON at 4°C. After washing 2 times with PBS, the sera samples were loaded in duplicate on the plate (50µL of 1:1000 dilution per sample). The PC loaded on the first row on either side was a 7-point dilution of ApoA1 (Sigma A0722) starting at 1.5µg/mL. The loaded plate was incubated for 1h at 37°C and then washed 3 times with PBS-0.1% Tween. To each well, 50µL of primary antibody (goat anti-human ApoA1 - Ab 7613) diluted 1:5000 in 1%BSA-PBS 0.1% Tween were added and the plate left to incubate for at 37°C for 30min. Following 3 washes with PBS-0.1% Tween, 50µL per well of the secondary antibody (rabbit anti-goat IgG-HRP conjugate - Dako P0160) diluted 1:5000 in 1%BSA in PBS-0.1% Tween were loaded. After a 30min incubation at 37°C, the plates were washed 3 times with PBS-0.1% Tween and 50µL per well of the HRP substrate added and allowed to incubate for 5min at RT until colour change had occurred. At this point, 50µL of TMB stop solution was added to each well and OD read at 450nm.

Figure 13 illustrates the typical standard curves for the NApoA1 (A) and TApoA1 (B) ELISA protocols. The TApoA1 ELISA was the only one for which each point of the standard curve corresponds to a known ApoA1 concentration because pure commercial ApoA1 was the positive control for this assay. Therefore, for this assay only, instead of AU, concentrations in ng/mL are shown. In the Results section, mg/dL was the chosen unit to express TApoA1 concentration as it is the routinely used in clinical practice.

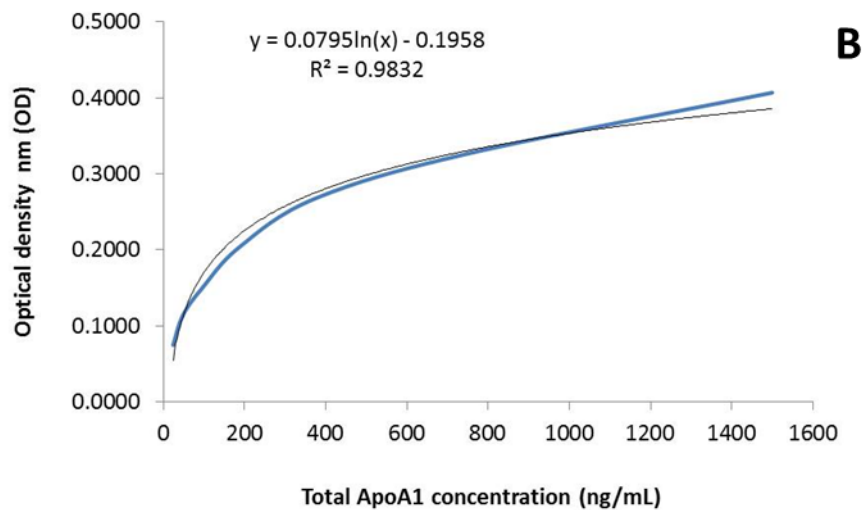
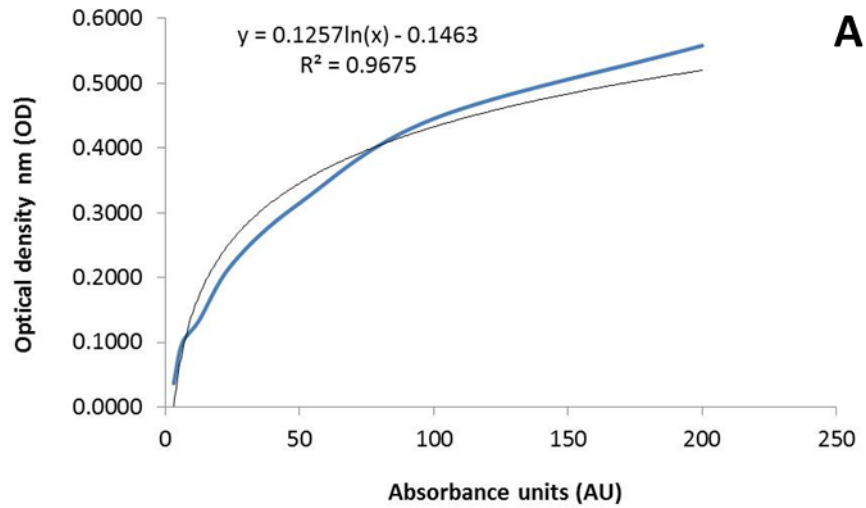


Figure 13: Standard curves for the NApoA1 (A) and TApoA1 (B) ELISA protocols. A pooled sera sample was used as a PC for the NApoA1 assay and absorbance units (AU) determined as previously described. In the TApoA1 ELISA, the standard curve was obtained using serial dilutions of human ApoA1. Consequently, each point of this curve corresponds to a known ApoA1 concentration (ng/mL) ($R^2 > 0.95$).

3.4: Vascular ultrasound of carotid and femoral arteries

3.4.1: Vascular US hardware and software specifications

The ultrasound equipment use was the Philips IU22. The probes used were the L9-3 MHz for the 2D imaging and the VL 13-5 MHz for the 3D assessment.

IMT measurements were performed using QLAB Advanced Quantification Software® version 7.1 (Philips Ultrasound, Bothell, USA). The same software was used to do the 3D US assessment and select 2D images for further image analysis.

For plaque area and texture assessment, Plaque texture analysis software – image analysis program for ultrasonic arterial wall changes and atherosclerotic plaques - version 4.5 (LifeQ Ltd Copyright 2013) was used. The same software was also used for the 3D plaque texture and volume analysis as described further in the Methods section.

3.4.2: Vascular US protocol

Patients lay on their back on an examination couch. The neck and groin were exposed and clothes protected with tissue paper. The examination was sequential and began by assessing the left carotid artery, followed by the right carotid artery, right common femoral artery and finally the left common femoral artery.

IMT measures were only considered on the carotid arteries (CCA and carotid bulb) and were always done in the posterior arterial wall. Measurement of the IMT on the CFA was done purely to determine inter-observer variability.

Whenever plaque was present, colour and Power Doppler images were obtained to ensure that the outline of the plaque was accurately determined. This was particularly important in echolucent plaques. Regardless of their location, all plaques were considered and thickness was measured using manual callipers.

Unless prevented by anatomical constraints, 3D US imaging was performed when carotid plaques were found. Plaques in the femoral arteries were not included in the 3D imaging protocol. Dr Maura Griffin did all the 3D US assessments after the 4 sites had been imaged.

At the end of this assessment, while the patient was still lying down, blood pressure (BP) was measured in the left upper arm using an automated BP reader (Omron M6 HEM-700-E). Three measurements were taken and the first discarded. The average systolic and diastolic BP value was calculated using the second and third readings. The mean BP value was calculated for each patient using the following formula:

$$\text{Mean BP} = \text{Diastolic BP} + 1/3(\text{systolic BP} - \text{diastolic BP})$$

The US protocol I used to scan all patients is as follows:

1) *Carotid US imaging*

An overall assessment of the whole length of the carotid artery from the proximal extremity of the CCA to the carotid bifurcation into ICA and ECA was obtained by sweeping the neck in 2D mode with the probe placed transversely. This was followed by retracting back from the carotid bifurcation to the proximal CCA in colour mode. This technique allows for a gross assessment of whether any changes (i.e. plaque) are present. The probe was then placed in the mid-section of the CCA and a longitudinal view was obtained. Once the artery was aligned (i.e. probe at a 90 degree angle thus showing clear differentiation between tissue interfaces), the image was zoomed in and a still picture was captured to allow for IMT measurement. After measuring the IMT, the image was un-frozen and a short video clip of the CCA was obtained for arterial wall motion analysis. The probe was then turned and a transverse view of the CCA was obtained. After aligning the vessel (i.e. showing IMT on the top and bottom of the artery), a measurement of the IMT was obtained. As in the longitudinal view, a video-clip of the CCA in transverse view was recorded.

After completing the assessment of the CCA, I moved up to the bulb. When possible, a longitudinal view of the carotid bifurcation was obtained, showing a small segment of distal CCA, the carotid bulb and both the ICA and ECA (Figure 14). To confirm the relative position of the ICA and ECA, an image of the first ECA branch, the superior thyroid artery, was always obtained even when it was not possible to get all the vessels on the same plane.

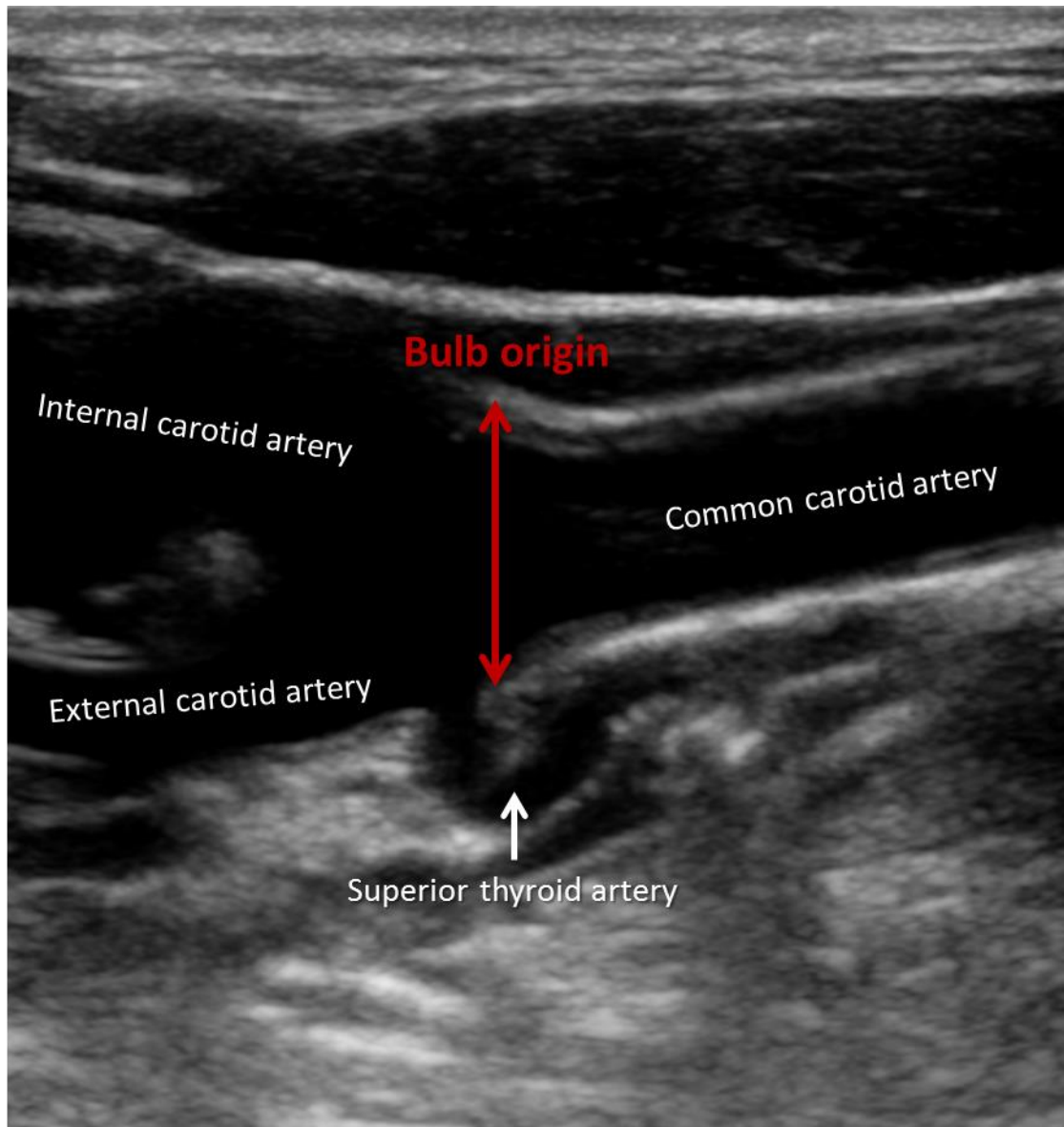


Figure 14: Longitudinal US view of the carotid bifurcation: the distal portion of the CCA gradually balloons to form the carotid bulb (signalled by the red line). Subsequently, the two branches formed after the first division of the CCA emerge: ICA, the ECA and its first branch, the superior thyroid artery.

When this was not possible due to anatomical variations, a longitudinal view of the internal carotid artery was obtained, including a portion of the CCA, carotid bulb and proximal segment of the ICA. Once the vessels were aligned, a still image was recorded and the IMT in the bulb origin was obtained. Bulb origin was defined as the area when the CCA starts to balloon out. To ensure that no plaque was present, colour and Power Doppler images of the carotid bifurcation were obtained showing the absence of filling defects. The US probe was then re-positioned: a transverse view of the bulb origin taken just before the carotid bifurcation was obtained and the IMT was again measured. If plaque was present, the IMT measurement in the bulb was disregarded and the plaque thickness was considered instead.

2) Femoral US

The probe was placed transversely in the groin area. After identifying the CFA, the artery was followed in transverse until the bifurcation became apparent. The probe was then turned and a longitudinal view was obtained which included the distal portion of the CFA and the division into deep and superficial CFA. When the position of the branches of the CFA prevented capturing a view of the bifurcation, an image of the CFA continuing into deep femoral artery was accepted. As described before, colour and Power Doppler images were also obtained. Finally, a transverse view of the CFA was obtained. No video-clips of the arterial motion were taken.

3.4.2.1: IMT measurement and characterization

The measurement of the IMT in the CCA was performed by using the automated software (QLAB). A longitudinal image of the CCA was obtained and transferred to QLAB. A section of 2cm was then selected when possible (>95% patients) and IMT measurement was accepted when accuracy was at least 97%. Measurement of the IMT of the CCA in the transverse view was noted by using manual callipers. This method was also applied to the measurement of both longitudinal and transverse views of the bulb.

With the exception of the patients selected for inter-observer variability assessment, IMT measurement on the CFAs was not obtained as there is no evidence for its clinical relevance.

3.4.2.2: 2D plaque analysis – plaque area and texture

For 2D plaque analysis, all plaques were considered regardless of their anatomical location and size.

Plaque type was established according to the visual scale after the images were reviewed by Dr Maura Griffin and I.

For the assessment of plaque texture, I used the Plaque texture analysis software – image analysis program for ultrasonic arterial wall changes and atherosclerotic plaques - version 4.5 (LifeQ Ltd Copyright 2013) as previously described. This software gives semi-automated measurements of plaque area (mm²) and GSM based on longitudinal images of each plaque. Before being analysed, each plaque imaged underwent normalization as previously described to ensure that all the images had homogenous settings.

3.4.2.3: 3D plaque analysis – plaque volume and texture

3D plaque analysis has only been done in carotid plaques. I identified 46 carotid plaques but had to exclude three from this part of the analysis as there were limitations to collecting 3D US plaque images. These include anatomical constraints (high carotid bifurcations with interposition of jaw) and presence of heavy calcification within the plaque.

The images for the volume analysis are collected by capturing a longitudinal view of the plaque using the 3D probe described above. The image analysis software (QLAB) then allows adjusting the image settings to a set standard and to “slice” the plaque, thus obtaining multiple transverse view cuts along the length of the plaque. The thickness of the slices is a key element in determining plaque volume and therefore needs to be recorded. The set slice thickness I used most commonly was 2mm but in smaller plaques, I decided to use 1 and 1.5mm slices so that each plaque had a minimum of 6 slices. Only the transverse slices where plaque was visualized were considered. After image normalization, area and GSM for each individual plaque slice or transverse view were determined.

Plaque volume was determined by using the following formula:

$$\text{Volume} = (\text{area1 (mm}^2\text{)} \times \text{slice thickness1 (mm)}) + \dots + (\text{area}\chi \times \text{slice thickness}\chi)$$

The use of 3D vascular US also permits to obtain a GSM value calculated from the different transversal slices obtained according to the method described above. As the slices go through the plaque, differences in plaque texture may become more apparent than when assessing the GSM based on a 2D image.

Volume-based GSM (GSMvol) was calculated by using the following formula:

$$\text{GSMvol} = \frac{(\text{GSM 1} \times \text{area 1}) + \dots + (\text{GSM } \chi \times \text{area } \chi)}{\text{area 1} + \dots + \text{area } \chi}$$

Thus, for every plaque, volume-based texture was obtained based on the GSM of each individual slice and its corresponding area.

3.4.3: Intra and inter-observer variability

In order to demonstrate the reproducibility of my results, inter and intra-observer variability was assessed. Inter-observer variability was determined by the sequential assessment of 25 patients scanned by both Dr Griffin and myself following the method described above. To ensure that no bias occurred, Dr Griffin repeated the measurements in reversed order after I had finished the scanning protocol. Intra-observer variability was determined in 10 patients who I scanned twice, once as described above and then in reversed order.

The same process was applied to the plaque area, plaque texture and plaque volume analysis to verify reproducibility of the image analysis methods.

Intra and inter observer variability was found to be <10% in both methods.

3.5: Statistical methods

In the longitudinal study, NN, NA and anti-NCS levels were assessed together as they were considered to be potentially correlated. The outcome variable for each individual analysis was NN, NA and anti-NCS level. An examination of the distribution of these variables indicated that it was highly positively skewed in its distribution. This was partly due to a large number of 0 values. Sometimes with positively skewed data it is possible to transform it to a normal distribution via the use of a transformation. However, the data in this case was too skewed for this to be possible. Therefore, an alternative approach was required. Instead of using statistical methods based on assuming a normal distribution, it was assumed that the outcome followed a 'negative binomial' distribution. This is a distribution suitable for such skewed distributions. A further feature of the data is that there were multiple samples from each patient. Standard statistical methods assume that each individual observation is independent of each other. This is unlikely to be the case when there are repeated samples, as samples from the same subject are likely to have more similar outcomes than samples from different patients. To allow for this structure of the data, multilevel statistical methods were used for the analysis. Two-level models were used with individual measurements clustered within patients. As a result of both the structure of the data and the distribution of the outcome, all analysis was performed using multilevel negative binomial regression. The analysis was performed in two stages. Firstly the separate effect of each factor upon the

outcome was examined separately in a series of univariable analyses. For explanatory variables measured on a continuous variable the shape of the relationship between each factor and the outcome was examined to see if a 'linear' relationship was appropriate, or whether a more curved relationship was more appropriate. One explanatory variable, dsDNA, was found to have a much skewed distribution, and so was analysed on the log scale. Subsequently the joint effect of the factors was examined in a multivariable analysis. This had the advantage that the effect of each factor upon the outcome is adjusted for the effects of the other variables, and so gives a better idea of the underlying factors associated with the outcome. To restrict the amount of variables in this second stage of the analysis, only factors showing at least some evidence of an association with the outcome in the univariable analysis ($p < 0.2$) were considered for this part of the analysis.

Before considering variables for the multivariable analysis, the collinearity between variables was examined. Collinearity is a statistical problem which occurs when you have highly correlated explanatory variables. This can cause problems with the fit of the regression models. Variance inflation factors (VIFs) were used to help assess this.

In the multivariable analysis, a backwards selection procedure was employed to retain only the statistically significant variables. This involves removing non-significant variables, one at a time, until all remaining variables are statistically significant.

Initially the separate effect of factor upon NN, NA and anti-NCS levels was examined in a series of univariable analyses. Due to the statistical methods used, the results are reported in terms of ratios. This gives the ratio of each variable values in one situation relative to another. For variables measured on a categorical scale, this represents the ratio of NN, NA and anti-NCS values in each group relative to the values in a baseline group. For variables measured on a continuous scale, these represent the relative change in the outcome level for a one-unit increase in that variable (ratios for differing sizes increases are reported where one-unit was either a very small or very large amount). In all instances a ratio of greater than 1 would suggest an increased NN, NA or anti-NCS level, whilst a ratio of less than 1 would suggest a lower level.

A similar methodological approach was used to analyse the results from the longitudinal assessment of anti-ApoA1. All the statistical analysis done for the longitudinal group, including the multivariable analysis was done using the statistical software Stata® (StataCorp LP, Texas, USA) by a statistician, Dr Paul Bassett.

The univariable statistical analysis for the cross sectional assessment in the vascular ultrasound and early disease cohorts as well as the individual variable assessment on the

longitudinal cohort was done by me using Prism® (GraphPad Software, La Jolla, California, USA). Given the variables, namely anti-ApoA1 levels did not follow a normal distribution, non-parametric tests were performed: the Mann-Whitney test was used when comparing two variables and the Kruskal-Wallis test when comparing more than two variables. Statistical significance was considered when p value was less than 0.05. In the longitudinal group, in order to overcome clustering considering that repeated measures for each patient were used, the mean overall value was used for each individual patient. Kaplan-Meier analysis to assess the impact of anti-apoA1 positivity on mortality in the early SLE cohort was done by my supervisor Anisur Rahman and later reproduced by me under his guidance. I supervised a BSc student Maria Davari during her project which focused on anti-apoA1 measurement during early SLE and part of this univariate analysis was done by her under my supervision.

Finally, when assessing the vascular US group, univariable analysis was done by me using both Prism® (GraphPad Software, La Jolla, California, USA) and SPSS 22® (IBM Corp, New York, USA). For variables with a normal distribution, parametric tests were used; otherwise, non-parametric tests were selected. To investigate correlations between variables, Spearman's correlation was used when distribution was not found to be normal and Pearson's correlation coefficient when distribution was normal, using 95% CI on both instances. r^2 values equal or greater than 0.2 were considered provided that the p -value was less than 0.05.

When assessing multiple comparisons, for instance, when comparing the IMT thickness measured at the right and left CCA for each individual patient, the Bonferroni correction was applied so that clustering could be minimized. This method was used to correct all the p values for multiple comparisons as follow-up test to ANOVA.

A non-SLE cohort was selected based on population studies as previously described. All the statistical analysis comparing this group with the SLE cohort was done by Professor Andrew Nicolaides using SPSS 22® (IBM Corp, New York, USA) and later reproduced by me under Dr Griffin's supervision.

Multivariable analysis was not performed in the vascular US cohort as, after discussing with the statistician, I realized that in order to achieve significant statistical power at least ten patients for each variable considered would be required. Given that I had a limited number of patients I opted for doing an exploratory analysis using univariate statistical methods. This means that although my analysis is underpowered by the size of the sample considered compared with the number of variables included; I was able to explore the potential correlations/ associations between them.

A final note concerns the usefulness of age adjusted statistical analysis, particularly when considering the vascular ultrasound group. Age adjustment is a statistical process applied to rates of disease, death or other health outcomes (i.e. individual variables) which allows for a fairer comparison between groups with different age distributions, as it renders them more comparable. The age-adjusted rates are those which would be have existed if the study population had a similar age distribution of a “standard” population previously defined.

I consider by performing age-adjustment and power calculations would have improved the statistical analysis of my thesis. Unfortunately, I was not able to perform these two important statistical tests in a useful timeframe as I do not have possess the necessary software or statistical skills which would have allowed me to do it on my own, nor was I able to procure a statistician who could help me to do this and I acknowledge this as a flaw in my statistical methods.

4: RESULTS

4.1: Overall population characterization

The longitudinal cohort of patients with lupus included 40 women and 9 men with a mean age at the time of the first sample of 36 years (SD 13.0). The ethnic distribution mirrored that of our clinic: 47% Caucasian ($n= 23$), 36% Afro-Caribbean origin ($n= 18$) and 16% ($n= 8$) had other ethnic backgrounds, namely Asian. The 37 healthy controls selected had a mean age of 31.6 years (SD 6.0), two thirds were female and 73% were Caucasian.

The early disease cohort included 499 subjects from which sera samples were taken within the first year following diagnosis. The mean age was 30 years (SD 12.27, range 1-77) and 91.5% were female. Regarding ethnicity, 2/3 of patients were Caucasian ($n= 316$), 20% were of Afro-Caribbean origin ($n= 106$) and 11% were South Asian ($n= 59$). A minority of patients were of East Asian descent ($n= 24$) or other ethnic origin ($n= 13$).

Regarding the disease control group, the mean age of the 17 patients with RA was 52 years (SD 15.8; range 22-87) and 71% ($n= 12$) were female, 76% ($n= 13$) were Caucasian, 18% ($n= 3$) Afro-Caribbean and 6% South Asian ($n= 1$). Serologically, 11 had seropositive RA (2 were anti-CCP positive and RF negative; 1 was anti-CCP negative but RF positive and 8 were positive both for anti-CCP and RF) and 6 were seronegative. Although data on disease activity score (DAS-28) were not available, patients were considered clinically to have active arthritis at the time of sample collection. No data on the treatment regimen at the time of sample were available. The small cohort of patients with myositis ($n= 12$) and Sjogren's syndrome ($n= 13$) who were tested for NN levels included, respectively, 2 males and 10 females (mean age: 48 years - SD 10.2; range 31-76) and 13 women (mean age: 54 - SD 16.7; range 32-81), all of which were Caucasian.

4.2: Protein nitration

In order to assess nitration levels, three protein targets were selected: nucleosomes, albumin and apolipoprotein A1. NN, NA (and anti-NCS) levels were tested in sera samples selected from the longitudinal cohort of patients with SLE.

NN, nitrated apoA1 and total apoA1 were tested in the cross-sectional group of patients who undergone vascular US (as described in section 3.1: Patient selection – Chapter 3: “Material and Methods”). I will only describe in detail the results obtained for the latter two assays. The findings for NN in the vascular US cohort were similar to those found for the longitudinal

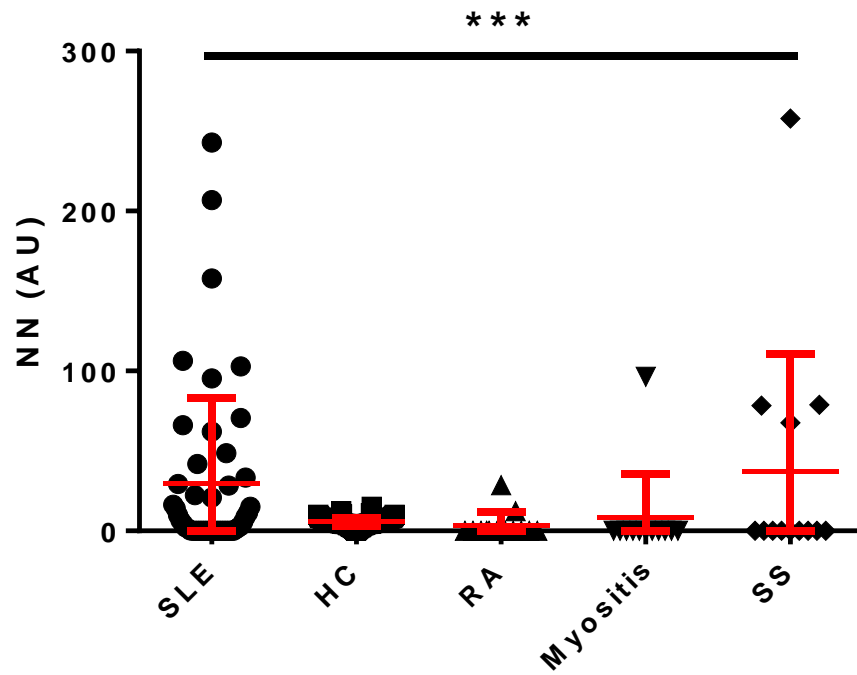
group, but given the smaller sample size and cross-sectional nature of this cohort, the data are poorer and no further relevant findings were noted. The results regarding the correlations between these two variables and imaging data will be discussed in Chapter 4.3: Vascular US.

4.2.1: Nitrated nucleosomes and nitrated albumin in the longitudinal cohort

The presence of NN was found in at least one occasion in 63% of the patients. Patients with SLE had significantly ($p= 0.0007$) higher NN levels compared with healthy controls and patients with other auto-immune diseases, namely RA, myositis and Sjogren's syndrome ($p<0.0001$) as shown on Graph 1.

18 patients never had detectable serum NN during the follow-up period considered, following a non-Gaussian distribution. In the 31 patients who had detectable NN, the levels varied widely over time (mean 32 AU; median 0.0; SD 62.2; IQR 0.0-30.5). Table 5 shows a comparison between characteristics of NN positive and NN negative patients. As shown in table 5, all the patients who were NN negative were anti-Sm negative (18/18) whereas 35% of the NN-positive patients were anti-Sm positive (11/31).

In the vascular US group, over a third of the patients ($n= 37$) were NN positive in their single samples. The results for NN values in the US group are not considered any further in this thesis. All the results on NN and NA described in the following sections refer to the longitudinal cohort.



Graph 1: Comparison between nitrated nucleosome (NN) levels in patients with SLE, healthy controls (HC), rheumatoid arthritis (RA), auto-immune myositis and Sjogren's syndrome (SS). Each point represents one patient for all columns. The column depicting patients with SLE includes the median NN level for each individual patient included in the longitudinal cohort to eliminate over-representation due to repeated samples per patient. (***) $p < 0.001$

Variables	NN-negative (n patients= 18) (n samples= 145)	NN-positive (n patients=31) (n samples= 252)
Mean Age at first sample(SD)	38 (13.1)	35 (12.8)
% Female (n)	77.8 (14)	83.9 (26)
Ethnicity		
% AC (n)	27.8 (5)	41.9 (13)
% C (n)	61.1 (11)	38.7 (12)
% Other (n)	11.1 (2)	19.3 (6)
Mean anti-dsDNA IU/ml (SD)	217.2 (428.3)	381.3 (1038.0)
Mean C3 g/l (SD)	1.0 (0.2)	0.9 (0.3)
Mean Global BILAG score (SD)	5.8 (4.8)	7.8 (6.9)
% anti-Sm positive (n)	0 (0)	35.5 (11)*
% anti-RNP positive (n)	27.8 (5)	32.3 (10)
% anti-Ro-positive (n)	38.9 (7)	41.9 (13)*
% anti-La positive (n)	16.7 (3)	9.7 (3)
% on hydroxychloroquine (n)	42.1 (61)	44.7 (113)*
% on immunosuppressants (n)	25.5 (37)	63.2 (160)*
% on ≥ 5mg/day prednisolone (n)	67.5 (98)	78.3 (198)
(* p< 0.05 on univariable analysis)		
Normal range for anti-dsDNA = 0 -50 IU/ml; for C3 0.9 – 1.8 g/L		

Table 5: Overall comparison between nitrated nucleosome (NN) positive and NN negative patients.
Variables found to reach statistical significance in the univariable analysis are highlighted in **bold**.

NN-positive patients appeared to have more active disease; with higher anti-dsDNA levels (381.3 vs. 217.2 IU/mL) and higher global BILAG score (7.8 vs. 5.8). Moreover, NN-positive patients were also more likely to be taking immunosuppressive drugs (63.2% vs. 25.5%) but this was also not statistically significant.

As shown in Table 6, the overall findings regarding the NA levels were similar to those found for the NN. 20 patients had detectable NA levels and 47.6% of the samples tested were positive for NA. NA levels did not follow a normal distribution. The majority of patients who were NA positive were female (16/20), the mean age was 34 (SD 11.4; 17-56) and the ethnic distribution was identical. In NA-positive patients, the levels varied over time (mean 28.8 AU; median 0.0 AU; SD 44.5; IQR 0.0-49.1).

Variable	Category / term	Ratio for NN (95% CI)	p-value for NN	Ratio for NA (95% CI)	p-value for NA
Gender *	Female (n= 336) Male (n= 61)	1 2.01 (1.06, 3.81)	0.03	1 5.00 (2.50,9.98)	<0.001
Disease duration †	Ratio given per 5- year increase	0.43 (0.23, 0.79)	0.005	0.32 (0.20,0.51)	<0.001
Age (at diagnosis) †	Ratio given per 10- year increase	0.10 (0.02, 0.46)	<0.001	0.04 (0.01,0.16)	0.002
Ethnicity*	Caucasian (n= 182) Afro-Caribbean (n=146) Other (n = 69)	1 0.75 (0.47, 1.22) 0.33 (0.17, 0.63)	0.003	1 2.07(1.26,3.40) 3.75 (1.86,7.56)	<0.001
Overall ENA**	No (n= 165) Yes (n= 232)	1 3.43 (2.19, 5.37)	<0.001	1 3.19 (1.93,5.27)	<0.001
	Anti-Ro** No(n= 226) Yes(n= 171)	1 1.70 (1.12, 2.59)	0.01	1 1.81 (1.14, 2.89)	0.01
	Anti-La** No(n= 345) Yes(n= 52)	1 0.79 (0.41, 1.50)	0.47	1 0.46 (0.2, 1.06)	0.07
	Anti-Sm** No(n= 298) Yes(n= 99)	1 5.63 (3.66, 8.67)	<0.001	1 6.25 (3.84, 10.2)	<0.001
	Anti-RNP** No(n= 288) Yes(n= 109)	1 1.08 (0.69, 1.70)	0.74	1 1.72 (1.06,2.79)	0.03
Anti-dsDNA level	< 50IU/ml (n= 177) ≥ 50IU/ml (n= 170)	1 0.76 (0.26, 1.13)	0.17	1 1.32 (0.66,2.65)	0.43
C3 level	< 0.9g/l (n= 139) ≥0.9g/l (n= 208)	1 0.83 (0.57, 1.20)	0.32	1 1.26(0.86,1.87)	0.24
Disease activity: (individual organ systems) General§	A, B (n= 31) C, D, E (n= 344)	1 0.96 (0.60, 1.54)	0.87	1 1.19 (0.71,2.00)	0.52
Mucocutaneous	A, B (n= 41) C, D, E (n= 334)	1 0.99 (0.63, 1.56)	0.95	1 1.32 (0.76,2.29)	0.33
Neuropsychiatric	A, B (n= 18) C, D, E (n= 357)	1 0.56 (0.13, 2.38)	0.44	1 1.04 (0.50,2.19)	0.91
Musculoskeletal	A, B (n= 47) C, D, E (n= 328)	1 1.20 (0.47, 3.04)	0.71	1 0.68 (0.43,1.05)	0.08
Cardiorespiratory	A, B (n= 13) C, D, E (n= 362)	1 0.43 (0.24,	0.006	1 1.40	0.72
Vasculitis					
Renal System					

Haematological		0.78)		(0.22,8.70)	
	A, B (n= 12) C, D, E (n= 363)	1 0.33 (0.17, 0.63)	0.001	1 0.39(0.19,0.82)	0.01
	A, B (n= 41) C, D, E (n= 329)	1 1.09 (0.40, 2.97)	0.86	1 4.28 (2.13,8.58)	<0.001
	A, B (n= 95) C, D, E (n= 280)	1 1.21 (0.84, 1.74)	0.30	1 1.44 (0.95,2.17)	0.08
Sustained disease activity	Persistently Low (n= 166) Persistently Mod /High (n= 209)	1 0.91 (0.66, 1.25)	0.57	1 0.66 (0.47,0.92)	0.01
Hydroxychloroqu ine	No (n= 223) Yes (n= 174)	1 3.52 (2.40, 5.17)	<0.001	1 3.21 (2.08,4.95)	<0.001
Immunosuppress ion	No (n= 200) Yes (n= 197)	1 1.66 (1.17, 2.34)	0.004	1 1.33 (0.89,1.98)	0.16
Prednisolone	≤7.5mg/day (n= 101) >7.5mg/day (n= 296)	1 0.81 (0.59, 1.13)	0.22	1 1.00 (0.69,1.44)	0.99
Albumin	Ratio given per 5g/l increase	1.38 (1.20,1.59)	<0.001	1.42 (1.22,1.65)	<0.001

*For gender, n values refer to the numbers of samples taken from female and male subjects, rather than numbers of females and males in the cohort of patients. A similar stipulation applies to ethnicity where n values refer to the number of samples taken from patients of each ethnic group.

† For the complex associations with disease duration and age, we carried out analysis of both linear and squared terms. The figures in the table refer to linear terms. The ratios for squared terms for NN are 0.66 (95% CI 0.51, 0.85) for disease duration and 1.56 (95% CI 1.20, 2.03) for age. The ratios for squared terms for NA are 0.59 (95% CI 0.46, 0.75) for disease duration and 1.72 (95% CI 1.32, 2.24) for age.

** For ENA, anti-Ro, anti-La and anti-Sm we did not have results from the date of every sample but it is assumed that the status of these antibodies generally remain stable

§ “Disease activity in general system” refers to the BILAG score (A, B, C, D or E) in the General

Category of the BILAG index on the day when each sample was taken. The same principle applies to all the other organ systems listed in the table, which are the 8 different categories recorded in BILAG.

Table 6: Univariable analysis of factors associated with serum nitrated nucleosomes (NN) and nitrated albumin (NA) levels in patients with SLE. Statistically significant p-values are highlighted in **bold.**

NN and NA levels were higher in men compared to women. For both outcomes a complex, non-linear relation with disease duration and age at diagnosis was found. NN and NA levels appear to be lowest in the youngest and oldest patients with a peak level at age 30 years. Similarly NN and NA levels are lowest for patients with the shortest and longest durations of disease with a peak level at around 8 years duration.

Although no statistically significant relation between both NN and NA and disease activity was found either serologically (high anti-dsDNA and/or low C3) or clinically (overall BILAG score), when considering the individual organ systems, the presence of vasculitis (i.e. score of A or B) was associated with significantly higher NA and NN levels. NN, but not NA, levels were significantly higher in cardiorespiratory flares. NN levels also appeared to correlate with neuropsychiatric disease as patients who scored C, D or E in this system appeared to have lower NN compared to those with an A or B score. However, this difference did not reach statistical significance due to wide confidence intervals. No similar trend was observed when considering NA levels. There were no further associations between individual systems scores and either NN or NA levels.

Upon overall comparison, NN and NA levels appear to correlate closely, however, the univariable analysis showed a few differences. The multivariable analysis exposed these differences further and as shown in Tables 7 and 8, NN and NA correlate differently with the variables considered.

Variable (NN)	Category / term	Ratio (95% CI)	p-value
Age (at diagnosis) *	Linear term Squared term	0.02 (0.003, 0.11) 2.12 (1.64, 2.76)	<0.001
Anti-Sm	No Yes	1 6.31 (3.39, 11.7)	<0.001
Vasculitis	A, B C, D, E	1 0.40 (0.24, 0.69)	<0.001
Hydroxychloroquine	No Yes	1 1.96 (1.09, 3.53)	0.02
Immunosuppression	No Yes	1 2.96 (1.97, 4.46)	<0.001
*Ratio given for 10-year increase			

Table 7: Multivariable analysis of factors associated with serum nitrated nucleosome (NN) levels in patients with SLE.

Variable (NA)	Category / term	Ratio (95% CI)	p-value
Disease duration	Ratio given per 5-year increase	2.24 (1.55, 3.26)	<0.001
Age (at diagnosis) *	Linear term Squared term	0.01 (0.00, 0.04) 2.58 (1.86, 3.56)	<0.001
Ethnicity	Caucasian Afro-Caribbean Other	1 2.99 (1.35, 6.65) 14.9 (5.82, 38.0)	<0.001
Anti-La	No Yes	1 0.16 (0.06, 0.49)	<0.001
Anti-Sm	No Yes	1 28.2 (14.4, 55.3)	<0.001
Renal involvement	A, B C, D, E	1 3.78 (2.07, 6.90)	<0.001
Immunosuppression	No Yes	1 1.45 (1.02, 2.05)	0.04
Albumin	Ratio per 5g/l increase	1.12 (1.01, 1.24)	0.03
* Ratio given for a 10-year increase			

Table 8: Multivariable analysis of factors associated with serum nitrated albumin (NA) levels in patients with SLE.

Age at diagnosis, disease duration, anti-Sm positivity and treatment with immunosuppressives have a similar association with NN and NA. Anti-Sm positivity in particular has a remarkably strong independent association with higher NN and NA levels ($p < 0.001$).

However, whilst NN appear to correlate independently with vasculitis and treatment with HQ, NA is associated with anti-La negativity, albumin and absence/low active renal disease.

4.2.2: Anti-nucleosome antibodies

The cut-off for positivity was defined as the 97.5th percentile of the healthy controls (0.17 AU). 85% samples were positive for anti-NCS and levels varied over time (mean 55.6 AU; median 10.6 AU; SD 94.5; IQR 0.0-77.4). The positive findings of the univariable analysis are shown in Table 9.

No associations were found between anti-NCS levels and sex, age and ethnicity. The ENA overall status and positivity for the different ENA subtypes were not associated with differences in anti-NCS levels.

Serologically, active disease defined by high anti-dsDNA and low C3 levels was associated with significantly higher anti-NCS levels ($p < 0.0001$ and $p = 0.038$ respectively) as shown on Figure 15-A and B.

Clinically defined disease activity characterized by BILAG showed that higher disease activity at the time of the sample was associated with higher anti-NCS levels ($p = 0.024$). Similarly, persistently moderate-highly active disease was also associated with higher anti-NCS levels as depicted on Figure 15-C and D.

Variable (anti-NCS)	Number of samples (n)	Mean anti-NCS level (SD)	p-value (high vs. low) (CI 95%)
Sustained disease activity			
Persistently moderate/high	209	59.1 (99.4)	
Persistently low	166	49.1 (84.0)	0.0242 (1.12 – 2.59)
Global BILAG score			
<5	166	49.1 (84.0)	
≥5	209	59.1 (99.4)	0.0242 (1.07 – 2.43)
Anti-dsDNA			
High (>50 IU/mL)	177	78.5 (112.5)	
Normal (≤50 IU/mL)	170	37.5 (75.2)	<0.0001 (1.33 – 2.04)
Complement (C3)			
Normal (≥0.9g/L)	209	50.1 (90.4)	
Low (<0.9g/L)	138	68.9 (105.6)	<0.0001 (0.79 – 0.9)
Hydroxychloroquine use			
Yes	174	43.5 (76.9)	
No	223	65.2 (105.4)	0.0375 (1.17 – 2.34)
Prednisolone daily dose			
<5mg/day	209	40.2 (76.9)	
≥5mg/day	188	72.8 (108.4)	<0.0001 (2.4 – 5.17)

Table 9: Univariable analysis of factors associated with serum anti-nucleosome antibody levels in patients with SLE.

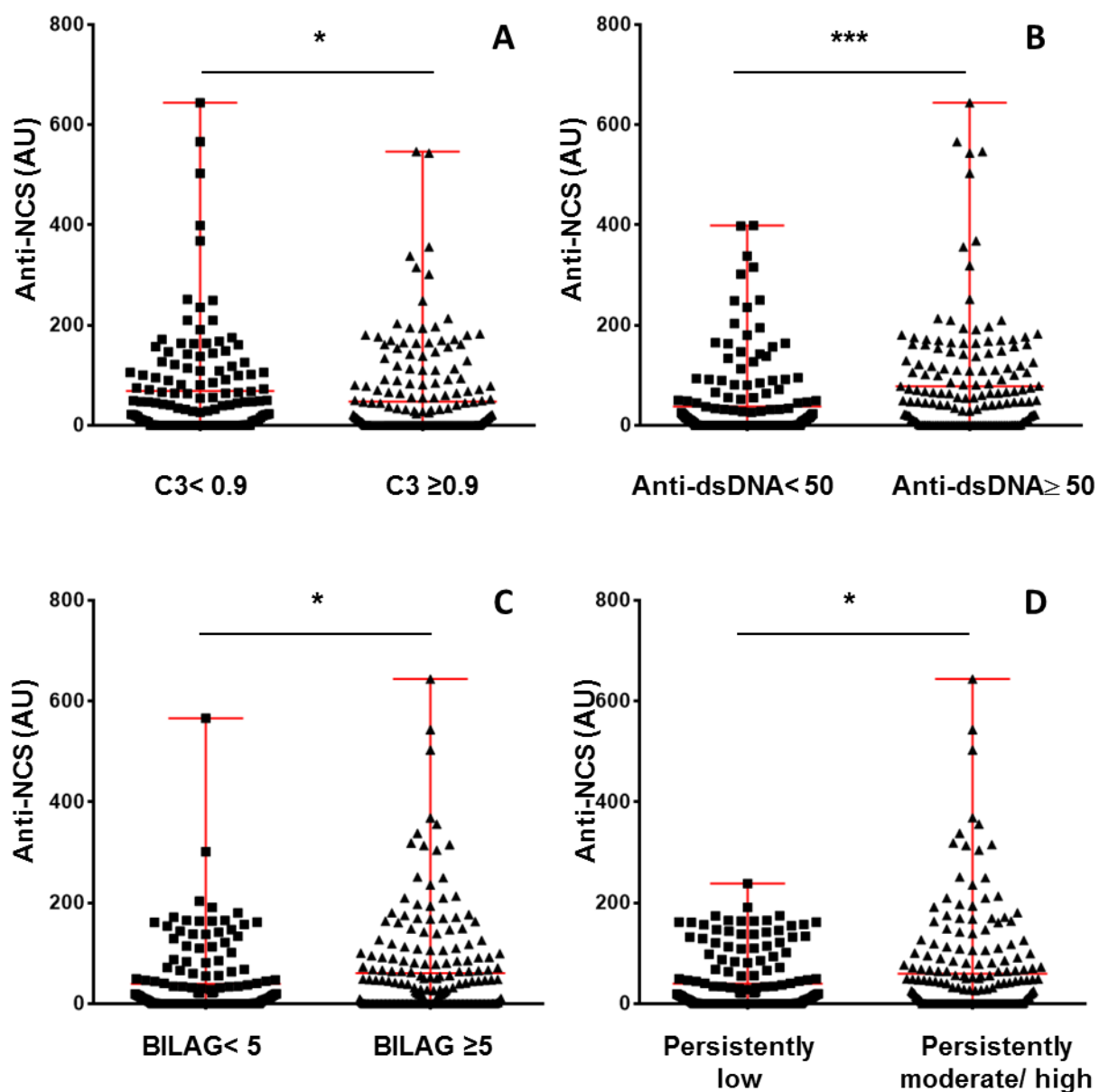


Figure 15: Anti-nucleosome antibody levels and measures of disease activity: comparison of anti-nucleosomes antibody levels and complement (C3) (A), anti-dsDNA (B), global BILAG score at the time of sample (C) and sustained disease activity (D)

(* $p < 0.05$; *** $p < 0.001$)

Treatment also appeared to influence anti-NCS levels. Patients who were taking HQ at the time the sample was taken had significantly lower anti-NCS levels than those who were not ($p < 0.0001$). Similarly, patients who were on low dose prednisolone ($< 5\text{mg}$ daily) at the time of sample had significantly lower levels of anti-NCS compared to those on a higher steroid dose ($p < 0.0001$) as shown on Figure 16. The use of immunosuppressants was not associated with differences in anti-NCS levels.

Multivariable analysis was also performed using a backwards selection procedure to retain only the statistically significant variables. The positive correlations elicited by the final regression model are summarised in Table 10.

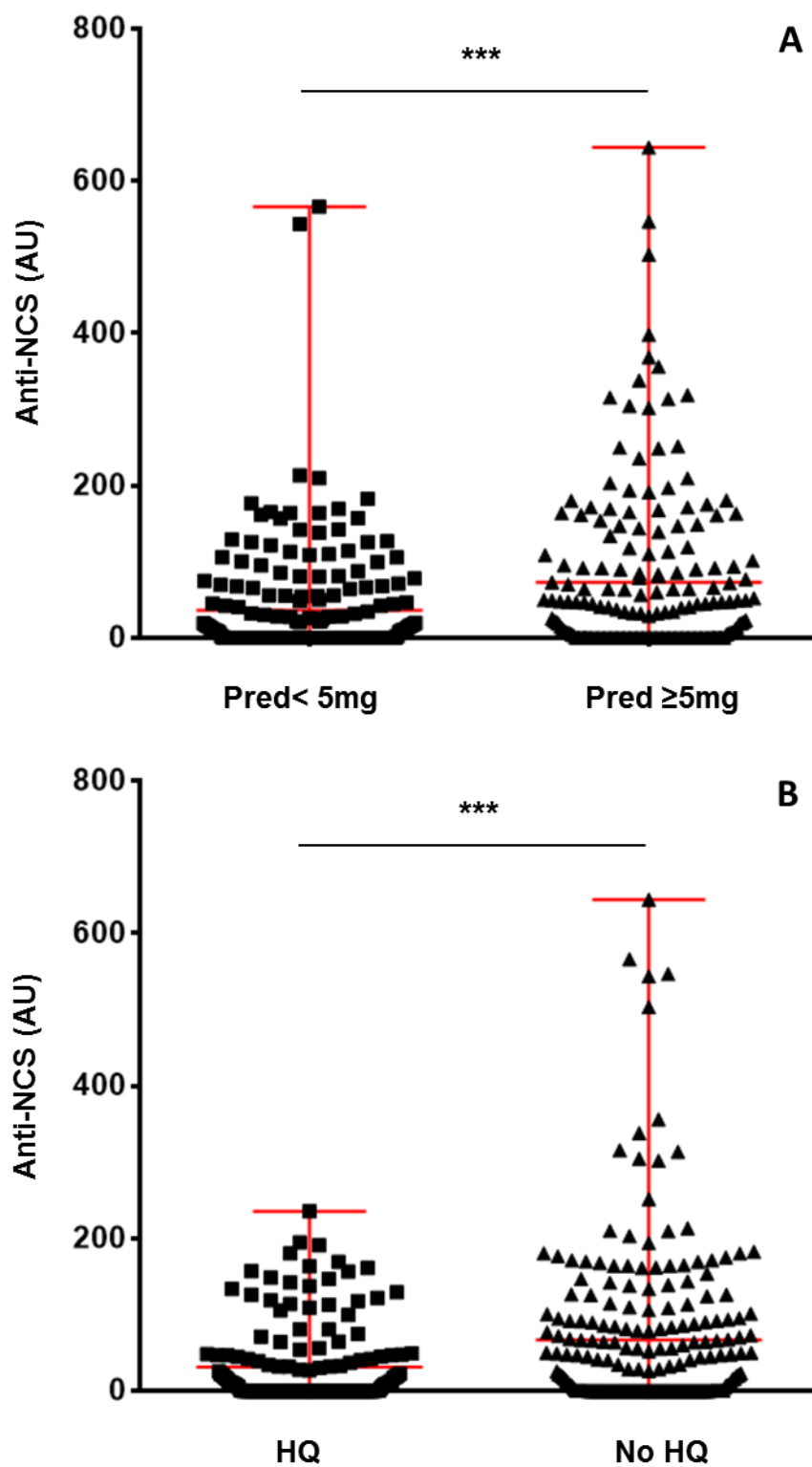


Figure 16: Anti-nucleosome antibody levels and treatment: comparison of anti-nucleosomes antibody levels in patients on low (pred< 5mg/ daily) vs. high dose prednisolone (pred ≥5mg daily) (A) and with or without treatment with hydroxychloroquine (HQ) (B).

(*** $p < 0.001$)

Variable (anti-NCS)	Ratio (95% CI)	P- value
Sex (male vs. female)	2.38 vs. 1 (1.72 – 4.69)	<0.001
Disease duration (**)	0.87 (0.78 - 0.98)	0.02
Age at diagnosis (***)	0.59 (0.49 – 0.72)	<0.001
RNP (neg vs. pos)	1 vs. 0.47 (0.32 – 0.7)	<0.001
Anti-dsDNA (†)	1.79 (1.4 – 2.28)	<0.001
Complement – C3 (*)	0.29 (0.15 – 0.56)	<0.001
(†) variable analysed on the log scale (base 10) (*) ratios given for a 0.1 unit increase in C3 level (**) ratios given for a 5 year increase in disease duration (***) ratios given for a 10 year increase in age		

Table 10: Factors found to significantly influence serum anti-nucleosomes antibodies (anti-NCS) levels in patients with SLE in a multivariable analysis.

Males were found to have anti-NCS levels almost 3 times higher than females. Both greater disease duration and older age at diagnosis were found to be associated with lower anti-NCS levels. A 5-year increase in disease duration was associated with a 13% reduction in anti-NCS levels, whilst a 10-year increase in the age at diagnosis was associated with a 40% reduction in anti-NCS.

Although the overall ENA status did not influence anti-NCS, anti-RNP positivity was associated with a 50% reduction in anti-NCS levels.

Similar to the univariable analysis, multivariable analysis confirmed a correlation between serologically active disease (defined by high anti-dsDNA and low C3 levels) and significantly higher anti-NCS levels. However, the multivariable analysis showed only a non-significant trend between higher clinically defined disease activity and higher anti-NCS levels. No association between the type of treatment and anti-NCS was found.

Finally, no association between NN and anti-NCS was shown and the two variables appear to vary independently of each other.

4.2.3: Nitrated apolipoprotein A1

The levels of nitrated ApoA1 measured in the sera of the patients included in the vascular US did not follow a normal distribution, hence all statistical analysis was performed using non-parametric tests. Patients with SLE had significantly higher levels of nitrated ApoA 1 compared with HC (median 32.1 vs 20.1; IQR 12.3 - 11.5 vs. 11.2 - 36.12). The positivity cut-off was defined as the 97.5th percentile of the HC (371.4) and 12 patients with SLE had nitrated ApoA1 levels above this threshold. No statistically significant associations between nitrated ApoA1 and disease activity were found. However, a non-significant trend was observed with clinically defined active disease as shown on Figure 17-A. Neither complement (C3) nor anti-dsDNA levels correlated with nitrated ApoA1 (Figure 17-B and C respectively).

No significant associations between nitrated ApoA1 and demographics, treatment and autoantibody profile (including anti-HDL and anti-ApoA1 IgG) were noted. NN and nitrated ApoA1 levels appeared to correlate as shown on Graph 2. Both variables were raised in patients with SLE compared with HC as illustrated on Figure 18.

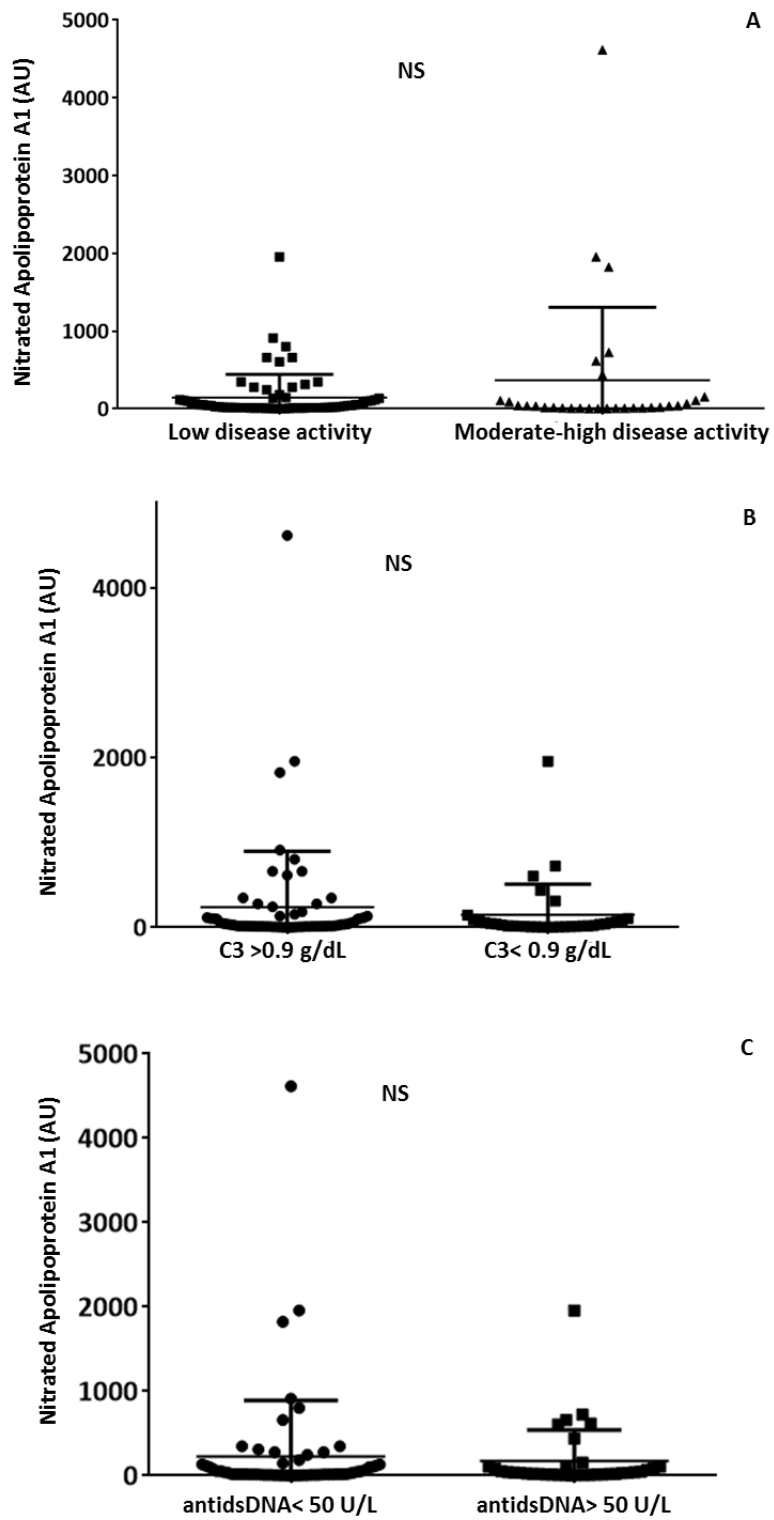
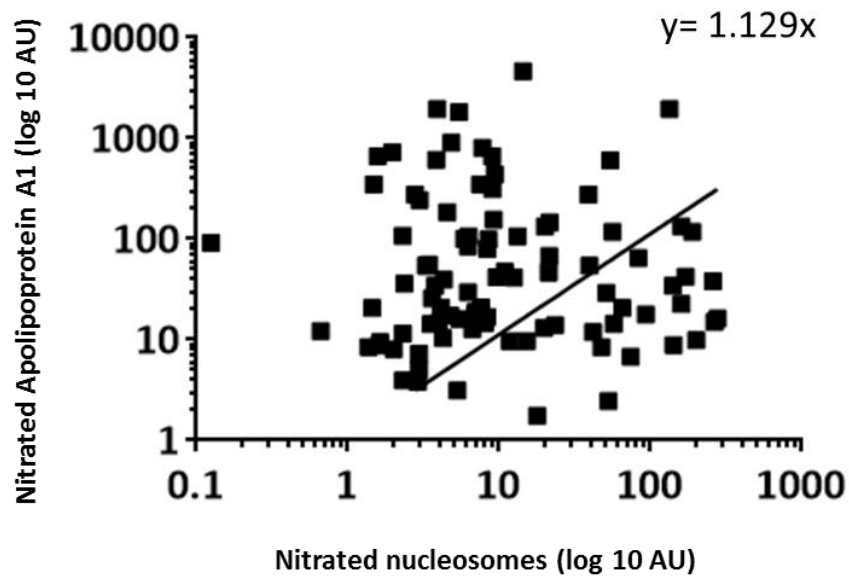


Figure 17: Nitrated ApoA1 levels and disease activity: nitrated apolipoprotein A1 levels in low (overall BILAG < 5) vs. moderate-high (overall BILAG ≥ 5) disease activity (A); low vs. normal complement (B) and normal vs. high anti-dsDNA antibodies (C) (NS: non-significant).



Graph 2: Correlation between nitrated nucleosomes and nitrated apolipoprotein A1 levels. The line depicts the linear regression and its equation ($n = 100$, $r^2 = 0.37$, $p = 0.06$).

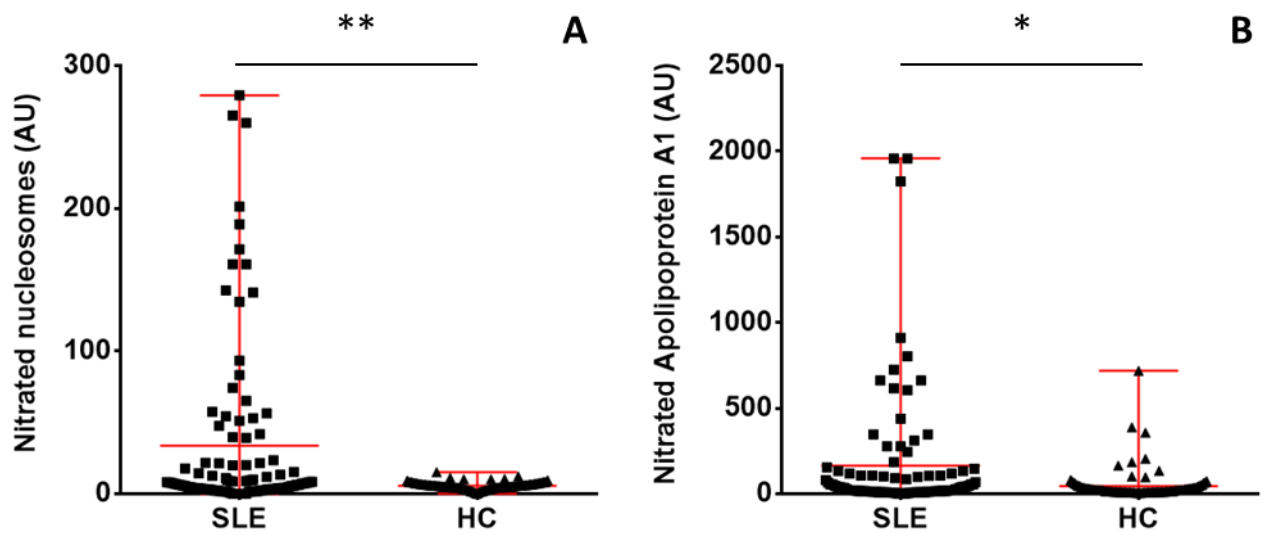
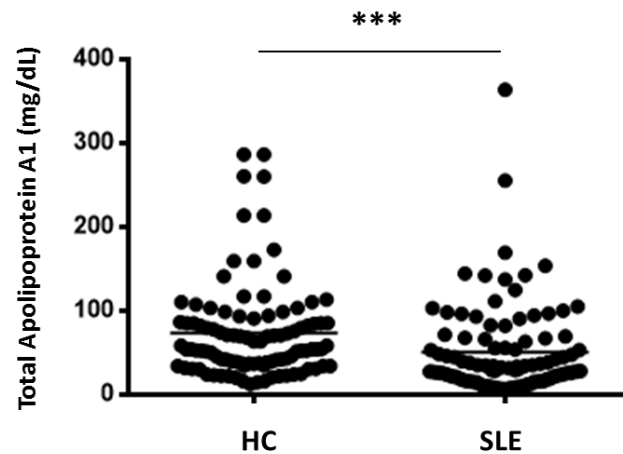


Figure 18: Comparison of nitrated nucleosomes (A) and nitrated apolipoprotein A1 (B) levels between patients with SLE and HC.

(* $p < 0.05$; ** $p < 0.01$)

In order to fully assess the impact of nitration of ApoA1, I determined the concentration of total ApoA1 on the same sera samples. Neither nitrated Apo A1 nor TApoA1 followed a normal distribution and therefore, all statistical analysis was done using non-parametric tests. The median and IQR for patients with SLE and HC was 31.1 vs. 54.3 and 17.8 – 363.7 vs. 36.9 – 286.5 respectively. As depicted on Graph 3, patients with SLE had significantly lower TApoA1 levels compared with HC.

Moreover, the correlation between TApoA1 and nitrated ApoA1 is not the same in patients with SLE and HC. As shown on Figure 19, in patients with SLE higher TApoA1 levels appear to correlate with lower nitrated ApoA1 titres whereas in HC, the correlation between these two variables is constant regardless of TApoA1 concentration.



Graph 3: Comparison of total apolipoprotein A1 levels between patients with SLE and HC.
 (***) $p < 0.001$

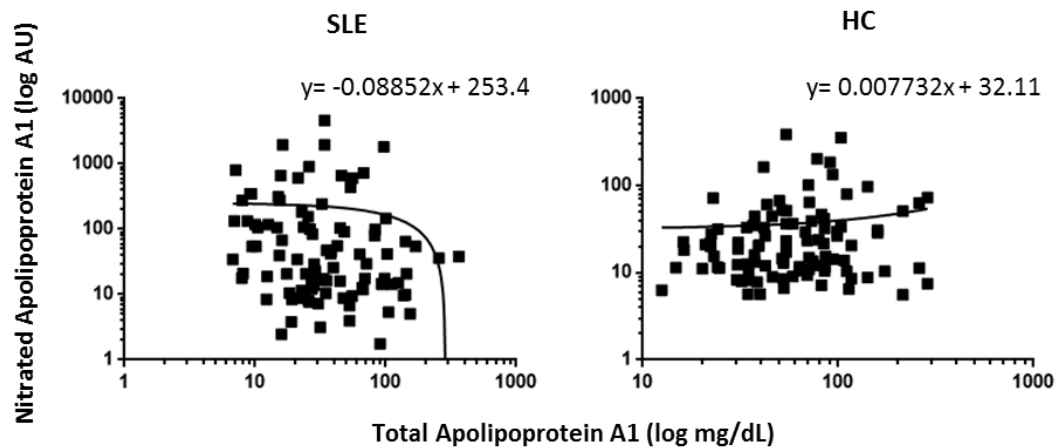


Figure 19: Comparison of the correlation between total and nitrated apolipoprotein A1 in patients with SLE and HC. The line depicts the linear regression curve and its equation (SLE $n = 100$, $r^2 = 0.19$, $p = 0.1$; HC $n = 100$, $r^2 = 0.12$, $p = 0.17$)

Considering that apoA1 may potentially be an antigenic target given the presence of anti-HDL and, in particular anti-ApoA1 antibodies, I assessed the correlation between these variables.

When plotting TApoA1 against anti-ApoA1 and anti-HDL IgG levels I obtained the graphs depicted in Figure 20. While anti-ApoA1 IgG levels did not seem to influence TApoA1 (Figure 19 – A), high levels of anti-HDL IgG were associated with a slight trend towards a decrease in TApoA1 (Figure 19 – B). This seems to suggest that anti-HDL but not anti-ApoA1 antibodies may lead to a decrease in serum TApoA1.

To further investigate this hypothesis, I defined four comparison groups: low and high anti-ApoA1 IgG levels ($\leq 25^{\text{th}}$ percentile and $\geq 75^{\text{th}}$ percentile, i.e. ≤ 31.1 and ≥ 181.0 respectively); low and high anti-HDL IgG levels ($\leq 25^{\text{th}}$ percentile and $\geq 75^{\text{th}}$ percentile, i.e. ≤ 0.0 and ≥ 24.86 respectively). I then applied a non-parametric T-test (Mann-Whitney) to compare each paired group, i.e. low vs. high anti-ApoA1 IgG and low vs. high anti-HDL IgG. My findings are summarized in Graph 4 and show that while there was no difference in TApoA1 levels between low vs. high anti-ApoA1 IgG antibodies, the presence of high anti-HDL IgG levels was associated with significantly lower TApoA1 levels ($p = 0.006$), thus supporting the hypothesis raised.

No further statistically significant associations were found between TApoA1, demographics, other autoantibodies, disease activity, treatment and lipid profile, including HDL levels.

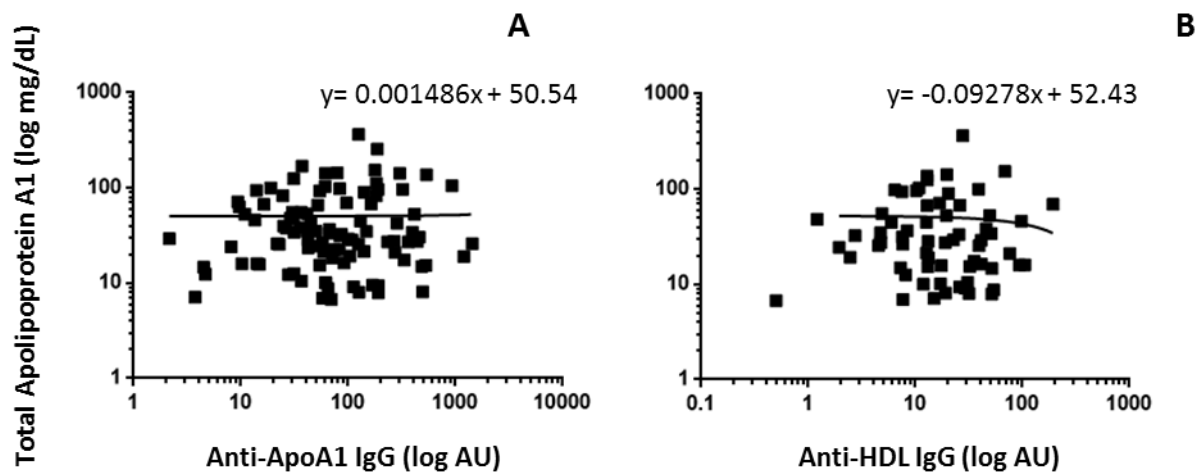
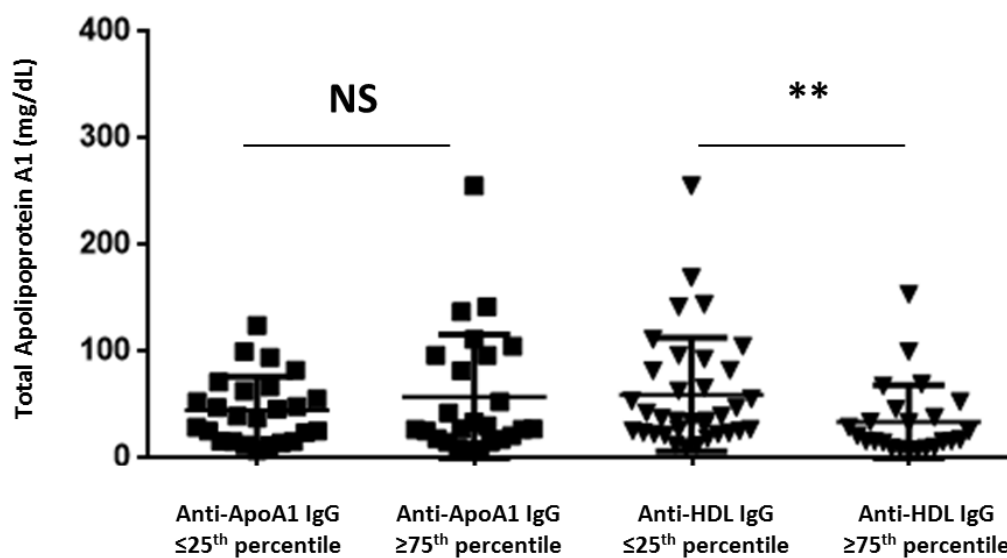


Figure 20: Comparison of the correlation between total apolipoprotein A1 and either anti-ApoA1 IgG (A) or anti-HDL IgG antibodies (B). The line depicts the linear regression curve and its equation (A $n = 100$, $r^2 = 0.02$, $p = 0.4$; B $n = 100$, $r^2 = 0.09$, $p = 0.1$).



Graph 4: Comparison of total apolipoprotein A1 concentrations in patients with low ($\leq 25^{\text{th}}$ percentile) vs. high ($\geq 75^{\text{th}}$ percentile) levels of anti-ApoA1 and anti-HDL IgG antibodies. (** $p < 0.01$)

4.2.4: Discussion

Nitric oxide metabolism has important implications in regulating endothelial function, with both protective and pathological properties and has been proposed as a potential mechanism for the generation of autoantibodies as a result of post-translational modification of self-antigens (Ahsan, Ali et al. 2003, Anderton 2004, Ohmori and Kanayama 2005). Additionally, the presence of enhanced nitrosative stress may lead to increased apoptosis, thus contributing to the accumulation of cellular debris which may then lead to auto-reactive processes and ultimately to the generation of autoantibodies (Estevez and Jordan 2002, Kim, Kim et al. 2002, Kim, Kwon et al. 2002, Duan and Chen 2007).

Several papers report on the presence of associations between NO synthase polymorphisms and SLE (Oates, Levesque et al. 2003, Serrano, Paez et al. 2004), the role of NO modified DNA and serum proteins in the pathogenesis of SLE and APS (Dixit and Ali 2004, Khan and Ali 2006, Ames, Batuca et al. 2010).

I chose serum nucleosomes as a potential target for nitration and developed a novel capture ELISA to measure the levels of NN. I found that mean NN levels are significantly higher in patients with SLE than in healthy controls, but there is a subset of patients with SLE who never test positive for NN. These persistently NN-negative patients comprise about one-third of the total population and have lower disease activity and anti-dsDNA antibody levels and less use of immunosuppressants than the other two-thirds. NN-negative patients are all anti-Sm antibody negative.

A strong association between anti-Sm and nitration was confirmed by the multivariable analysis of factors associated with NN and NA levels. To the best of my knowledge, the association between nitrosative stress and anti-Sm positivity has not been described before in SLE or any other disease state. The Sm antigen is a complex of ribonucleoproteins found in the spliceosome (McClain, Ramsland et al. 2002) and anti-Sm antibodies are highly specific to SLE. Anti-Sm have been found in lupus nephritis renal biopsies (Mannik, Merrill et al. 2003) and some retrospective studies reported associations between anti-Sm positivity and psychosis (Tikly, Burgin et al. 1996) or nephritis (Alba, Bento et al. 2003). A large Chinese study compared 469 anti-Sm antibody positive and 1115 anti-Sm antibody negative patients with SLE (Ni, Yao et al. 2009). The anti-Sm positive patients had higher disease activity, higher anti-dsDNA antibody and lower C3 levels than anti-Sm negative patients. Vasculitis occurred more often in anti-Sm positive than in anti-Sm negative patients (13.7% vs. 7.4%, $p < 0.05$). Our recently published results show strong associations between NN levels, anti-Sm and vasculitis (Croca, Bassett et al. 2014).

Increased nitration may promote development of anti-Sm antibodies. Experiments on mice transgenic for the rearranged heavy chain variable region genes of a monoclonal anti-Sm antibody (Clarke 2008) showed that immature dendritic cells (DC) carrying surface Sm antigen interacted directly with B cells leading to the production of anti-Sm antibody. These DC obtained Sm antigen from apoptotic cell debris, which also contain nucleosomes complexed to High Mobility Group Box 1 (HMGB1) protein (Urbonaviciute, Furnrohr et al. 2008). HMGB1 can activate DC (Yang, Postnikov et al. 2012) and also promotes phagocyte NO production by phagocytes. Thus nucleosome/HMGB1 complexes could stimulate the DC-B cell interaction leading to anti-Sm antibody production as well as driving NO production. It is not known whether nitration of HMGB1 itself alters its pro-inflammatory properties, but a recent study has shown that altering the redox state of HMGB1 through the oxidation of free thiol cysteine residues to form disulfide bonds makes it more pro-inflammatory (Urbonaviciute, Furnrohr et al. 2008). If nitration of nucleosome/HMGB1 complexes likewise promotes their pro-inflammatory properties this would create a potential positive feedback loop, and this is a mechanism that warrants further exploration.

NA and NN levels were also associated with the use of immunosuppressants. This association is unlikely to arise simply from the fact that immunosuppressants are used more commonly in highly active disease, because persistent clinical disease activity, anti-dsDNA antibody and C3 levels were not associated with either NA or NN levels. The drugs themselves could promote nitration, but I know of no evidence supporting this. Cyclosporin can promote tyrosine nitration in endothelial cells (Redondo-Horcajo, Romero et al. 2010), but only two samples in this study were from patients taking cyclosporin and both were negative for NN. No differences between MMF and azathioprine were noted.

Vasculitis is a relatively uncommon manifestation of SLE (Ni, Yao et al. 2009). In multivariable analysis I found a statistically significant association of high NN (but not NA) with vasculitis based on results from 9 different patients over an 11- year period. Review of the medical records showed that all these patients had cutaneous vasculitis at the time of their flares. It is possible that raised NN levels in patients without visible vasculitis may be a marker of subclinical vascular activation. It will therefore be interesting to see whether NN levels are associated with objective measures of atherosclerosis such as carotid ultrasound (Roman, Shanker et al. 2003). Peluffo et al and Aslan et al revised the evidence supporting the role of protein tyrosine nitration in CVD pathology and describe how post translational protein modifications may lead to gain and/or loss of function and contribute to disturb vascular homeostasis and consequently increase the risk of CVD (Peluffo and Radi 2007, Aslan and Dogan 2011). This potential association is underlined by the fact that peroxynitrite, a powerful nitrating and oxidising agent, is generated in atherosclerotic

plaques. If such an association exists then NN levels may be relevant to assessment of CVD risk in patients with SLE.

In summary, by developing a novel assay to measure serum NN levels I have demonstrated that these levels are raised in patients with SLE compared to healthy controls. NN-positivity is strongly linked to anti-Sm antibody positivity and may be a marker for neuropsychiatric flares and vasculitis in patients with SLE. Further studies in larger numbers of patients with these manifestations are required. Our group has now tested samples obtained from the SLICC sera bank from patients with SLE who had had various neurological manifestations, some potentially correlated with SLE (neuro-SLE) and others thought to be unrelated to lupus. All the samples were age and sex-matched for patients with SLE who had never had neurological manifestations. Preliminary data shows that samples from some patients whose neurological symptoms were originally not thought to be due to SLE (i.e. headaches) have high titres of NN, thus raising the hypothesis that they could in fact be associated with increased nitrosative stress and disease activity (data not published).

As discussed in the introduction, the presence of apoptotic cellular debris may play a role in the development of the auto-antibodies which are one of the key features of SLE and the presence of apoptotic nucleosomes has been related to the presence of anti-NCS antibodies which have been described in SLE (Stemmer, Richalet-Secordel et al. 1996, Min, Kim et al. 2002) (Kalaaji, Fenton et al. 2007). Several studies suggest that they may be useful to establish diagnosis, particularly in patients who are anti-dsDNA negative (Bizzaro, Villalta et al. 2012). Data have been published suggesting a potential association with target-organ involvement, namely with the presence of lupus nephritis (Mortensen, Fenton et al. 2008, Manson, Ma et al. 2009, Mortensen and Rekvig 2009), and disease activity (Ravirajan, Rowse et al. 2001, Simon, Cabiedes et al. 2004). It has also been suggested that anti-NCS antibody level may be a useful biomarker to predict the occurrence of disease flares (Cozzani, Drosera et al. 2014).

The majority of the patients with SLE whose sera I tested were anti-NCS positive (85%), which is in keeping with the figures reported in the literature (Min, Kim et al. 2002, Simon, Cabiedes et al. 2004, Isenberg, Manson et al. 2007, Cozzani, Drosera et al. 2014). The presence of active disease, defined either clinically (based on the BILAG score) or serologically (anti-dsDNA and C3 levels) was found to correlate with disease activity, echoing data already published. The association between higher anti-NCS and higher anti-dsDNA levels was particularly robust as it was found to be significant in univariate and multivariate analysis emphasizing the strong link between these two autoantibodies (Saisoong, Eiam-Ong et al. 2006).

An association between lower anti-NCS levels and treatment with HQ and low corticosteroid dose (prednisolone <5mg daily) was found in univariate but not in multivariate analysis. There are no published data reporting on similar assessment of anti-NCS levels and treatment.

Nitration of serum proteins is a post-translational modification and it may be hypothesized that this could be a potential mechanism contributing to the development of auto-antibodies (Ahsan, Ali et al. 2003, Anderton 2004, Ohmori and Kanayama 2005). Thus, I investigated whether the presence of NN correlated in any way with the levels of anti-NCS but found that this was not the case, as both variables vary independently.

Finally, I selected ApoA1 as a potential target for nitration given its pivotal role in HDL function and consequently, its cardiovascular protective role. Zheng et al reported on ApoA1 as a selective target for MPO-catalysed oxidative modification in human atheroma, suggesting that this could potentially be a mechanism for HDL dysfunction (Zheng, Nukuna et al. 2004). Later studies performed by Shao et al showed that although ApoA1 was in fact a target for nitration and chlorination, only the later led to significantly impaired cholesterol efflux, thus minimizing the role of this post-translational modification in ApoA1 function (Shao, Bergt et al. 2005). Bakillah using both an ELISA and Western Blot method reported that subjects with low serum HDL had significantly higher serum nitrated ApoA1 levels compared with those whose HDL level was normal. Moreover, the degree of nitrated ApoA1 containing HDL was negatively correlated with levels of circulating ApoA1 (Bakillah 2009). Recently, Aslan et al revised several studies which assessed the *in vivo* and *in vitro* effects of ApoA1 nitration and concluded that ApoA1 is a clear target for tyrosine nitration and that there is sufficient evidence to support that nitrated ApoA1 could potentially be used as a CVD biomarker (Aslan and Dogan 2011).

I found that patients with SLE had significantly lower levels of TApoA1 and higher levels of nitrated ApoA1 compared with HC. A non-significant trend between moderate-high disease activity and higher nitrated ApoA1 levels was observed but no statistically significant associations with anti-dsDNA and C3 were noted. However, when I compared the linear regression model between TApoA1 and nitrated apoA1 between patients with SLE and HC, I found that although there was no correlation in the HC cohort, there was a trend for patients with SLE who had higher TApoA1 levels to have lower nitrated ApoA1. Parastatidis et al reported their findings from an *in vivo* study using ApoA1-deficient mice describing how in the presence of lower ApoA1 levels, nitrosative stress appeared to be enhanced (increased protein nitration), supporting a potentially antioxidant effect which would support a specific protective role of ApoA1 (Parastatidis, Thomson et al. 2007). My findings also appear to

support this hypothesis, but further studies are required to firmly establish this mechanism in humans. Vasquez et al assessed HDL-dependent cholesterol efflux and endothelial function in obese women (BMI ≥ 30) and found that there was a positive correlation between these two variables. Moreover, women with increased BMI also had higher nitrated ApoA1 levels and this was associated with reduced cholesterol efflux, suggesting that this post-translational modification may reduce ApoA1 ability to accept cholesterol from ABCA1 transporters of peripheral macrophages. Hence, raising the hypothesis that increased nitrated ApoA1 levels may be a biomarker of increased nitrosative stress in the obese state, even in the absence of diabetes or dyslipidaemia (Vazquez, Sethi et al. 2012)

Tyrosine residues are the preferred site for nitration (Ischiropoulos 1998, Radi 2004, Abello, Kerstjens et al. 2009) and I found that NN and nitrated ApoA1 levels were closely correlated as I had previously found for NN and NA, thus supporting the notion that nitration is a systemic process and therefore it affects several protein targets in a similar way.

To assess whether the presence of nitrated ApoA1 was associated with increased anti-ApoA1 and/or anti-HDL antibodies, I used a linear regression model and found that there was no correlation between nitrated ApoA1 and anti-ApoA1. However, although a similar no-correlation pattern was observed for anti-HDL antibodies, there appeared to be a trend for lower TApoA1 concentration for the samples which had the highest anti-HDL levels. This raised the hypothesis that high anti-HDL levels could be associated with decreased TApoA1 and this was confirmed when comparing the lowest and highest quartile of anti-HDL as a statistically significant difference was found.

In summary, ApoA1 plays a central role in HDL function and has important cardiovascular protective functions. Reduced levels of ApoA1 are associated with increased CVD and the presence of increased nitrosative stress and nitrated Apo1 may be associated with impaired function and consequently, increased CV risk. Patients with SLE have lower TApoA1 and higher nitrated ApoA1 than HC, regardless of disease activity, treatment or classical autoantibody profile (ENA, anti-dsDNA, C3). However, the presence of anti-HDL IgG in high titres appear to be associated with lower TApoA1 levels. A similar pattern was observed for anti-ApoA1 IgG but it did not reach statistical significance. This supports the hypothesis that the presence of anti-HDL antibodies may be associated with increased ApoA1 clearance, possibly due to the formation of immune complexes, which would then support the notion that these antibodies may contribute to the increased CVD risk found in SLE. The presence of high levels of nitrated ApoA1 may also be a mechanism of HDL dysfunction and it would be relevant to assess the correlation and potentially causal association between these two variables.

4.3: Anti-apolipoprotein A1 and anti-HDL antibodies

4.3.1: Anti-apoA1 IgG antibodies

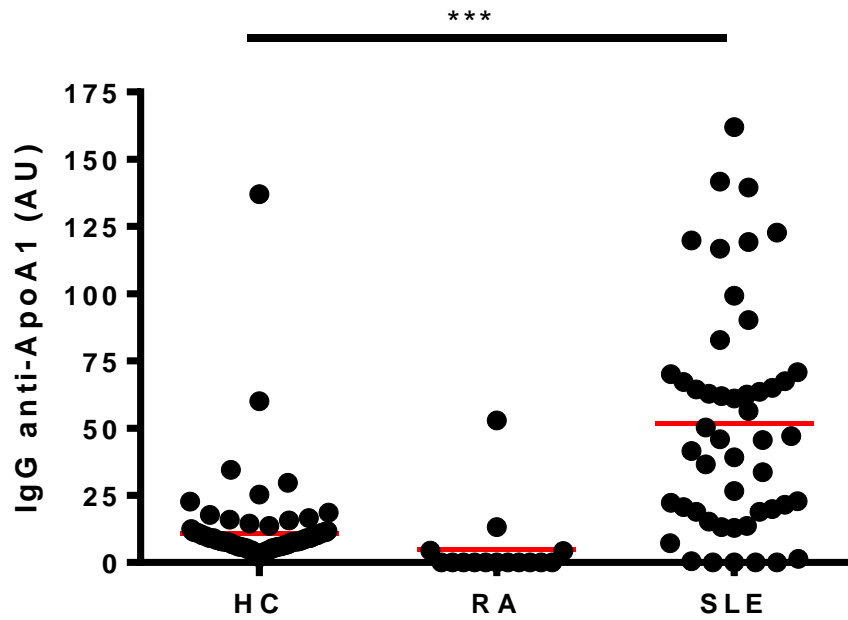
Anti-apoA1 IgG antibodies were tested in the longitudinal cohort and two separate cross-sectional cohorts (early disease and vascular US groups as described on section 3.1: Patient selection).

4.3.1.1: Longitudinal variation of anti-ApoA1 IgG levels

Considering the 397 samples included in the longitudinal cohort previously described, 50% (198/397) were positive for anti-ApoA1 IgG, defined as the 97.5th percentile of the 100 healthy controls (46.7 AU).

Anti-ApoA1 IgG levels were significantly higher ($p= 0.0001$) in patients with SLE (median 48.5; IQR 5.9-10.7) compared with healthy controls (median 8.0; IQR 5.9-10.7) and patients with RA (median 0.0; IQR 0.0-4.3) as shown on Graph 5.

The results of the univariable analysis between demographic and serological variables and anti-ApoA1 IgG are shown in Table 11.



Graph 5: Comparison of anti-ApoA1 levels between healthy controls (HC), patients with rheumatoid arthritis (RA) and SLE (each dot represents individual patients).

(*** $p < 0.001$)

Variable	Category	Ratio (95% CI)	P-value
Gender*	Female (n=336)	1	0.30
	Male (n=61)	1.20 (0.85, 1.69)	
Disease duration	Ratio given per five year increase	1.00 (0.90, 1.11)	0.99
Age at diagnosis	Ratio given per ten year increase	1.02 (0.88, 1.18)	0.81
Ethnicity*	Caucasian (n=182)	1	0.46
	Afro-Caribbean (n=146)	1.19 (0.90, 1.57)	
	Other (n=69)	1.07 (0.75, 1.53)	
Overall ENA status**	No (n=165)	1	0.13
	Yes (n=232)	0.82 (0.63, 1.06)	
Anti-Ro**	No (n=226)	1	0.41
	Yes (n=171)	0.90 (0.69, 1.17)	
Anti-La**	No (n=345)	1	0.002
	Yes (n=52)	0.54 (0.36, 0.80)	
Anti-Sm**	No (n=298)	1	0.004
	Yes (n=99)	0.64 (0.47, 0.87)	
Anti-RNP**	No (n=288)	1	0.46
	Yes (n=109)	1.11 (0.84, 1.47)	
Anti-dsDNA level	< 50IU/ml (n=177)	1	0.29
	≥ 50IU/ml (n=170)	1.13 (0.90, 1.43)	
C3 level	< 0.9g/l (n=139)	1	0.09
	≥0.9g/l (n=208)	0.82 (0.66, 1.03)	
Disease activity in general system §	A, B (n=31)	1	0.32
	C, D, E(n=344)	0.84 (0.60, 1.18)	
Disease activity in mucocutaneous	A, B (n=41)	1	0.48

system §	C, D, E (n=334)	0.89 (0.65, 1.22)	
Disease activity in neuropsychiatric system §	A, B (n=18) C, D, E (n=357)	1 0.93 (0.57, 1.52)	0.77
Disease activity in musculoskeletal system §	A, B (n=47) C, D, E (n=328)	1 0.80 (0.60, 1.08)	0.14
Disease activity in cardiorespiratory system §	A, B (n=13) C, D, E (n=362)	1 0.66 (0.42, 1.02)	0.06
Disease activity in vascular system §	A, B (n=12) C, D, E (n=363)	1 0.80 (0.47, 1.34)	0.40
Disease activity in renal system §	A, B (n=41) C, D, E n=329)	1 0.75 (0.55, 1.01)	0.06
Disease activity in haematological system §	A, B (n=95) C, D, E (n=280)	1 0.63 (0.50, 0.79)	<0.001
Sustained disease activity	Persistently low (n=166) Persistently moderate/ high	1 1.30 (1.06, 1.59)	0.01

	(n=209)		
Hydroxychloroquine	No (n=223) Yes (n=174)	1 0.70 (0.55, 0.88)	0.003
Immunosuppression	No (n=200) Yes (n=197)	1 0.90 (0.73, 1.12)	0.35
Oral prednisolone	≤ 7.5mg/day (n=101) > 7.5mg/day (n=296)	1 1.39 (1.13, 1.71)	0.002
<p>*For gender, n values refer to the numbers of samples taken from female and male subjects, rather than numbers of females and males in the cohort of patients. A similar stipulation applies to ethnicity where n values refer to the number of samples taken from patients of each ethnic group.</p> <p>** For ENA, anti-Ro, anti-La and anti-Sm we did not have results from the date of every sample but it is assumed that positivity and negativity for these antigens generally remain stable</p> <p>§ “Disease activity in general system” refers to the BILAG score (A, B, C, D or E) in the General Category of the BILAG index on the day when each sample was taken. The same principle applies to all the other organ systems listed in the table, which are the 8 different categories recorded in BILAG.</p>			

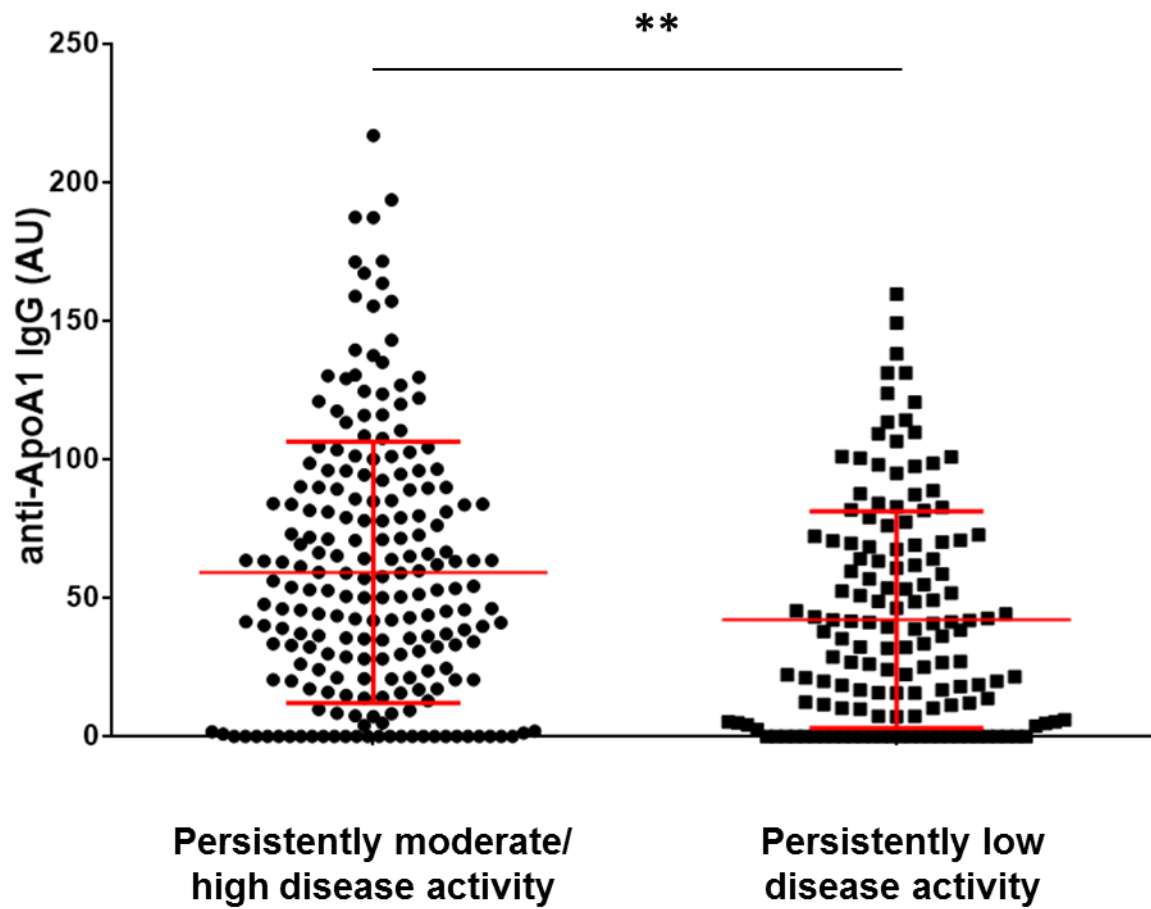
Table 11: Univariable analysis of factors associated with anti-ApoA1 IgG levels. The statistical significant associations are highlighted in **bold.**

No associations between anti-ApoA1 levels and either sex or ethnicity were found. Although anti-ApoA1 IgG levels were significantly lower in patients who were anti-La and anti-Sm positive, no statistically significant relationship was found with other serological markers, namely with C3 and anti-dsDNA which are the classical serological markers of disease activity.

Nonetheless, when considering clinically defined disease activity based on the BILAG score, I found that patients who had persistently moderate or high disease activity had anti-ApoA1 IgG levels 30% greater than those with persistently low activity (Graph 6).

Moreover, patients with haematological flares (i.e. BILAG score A or B in this system) had significantly higher anti-ApoA1 IgG levels than those with a score of C, D or E ($p < 0.001$). A similar finding was observed for patients with renal or cardiorespiratory scores of A or B compared with those who scored C, D or E but on both instances, statistical significance was not reached ($p = 0.06$). A summary of these findings is depicted in Figure 20.

The notion that anti-ApoA1 IgG levels appear to vary following a similar trend to that of disease activity, as defined by the BILAG, is further illustrated when individual patients are considered. Figure 22 depicts the variation of anti-ApoA1 IgG levels and global BILAG score in 8 patients. For the majority of them, these two variables appear to mirror each other (A-F) but for others, this variation is not as parallel (G-H).



Graph 6: Comparison of anti-ApoA1 IgG levels and sustained disease activity: patients with persistently moderate/ high disease activity have significantly higher anti-ApoA1 levels compared with those with persistently low activity.

(** $p < 0.01$)

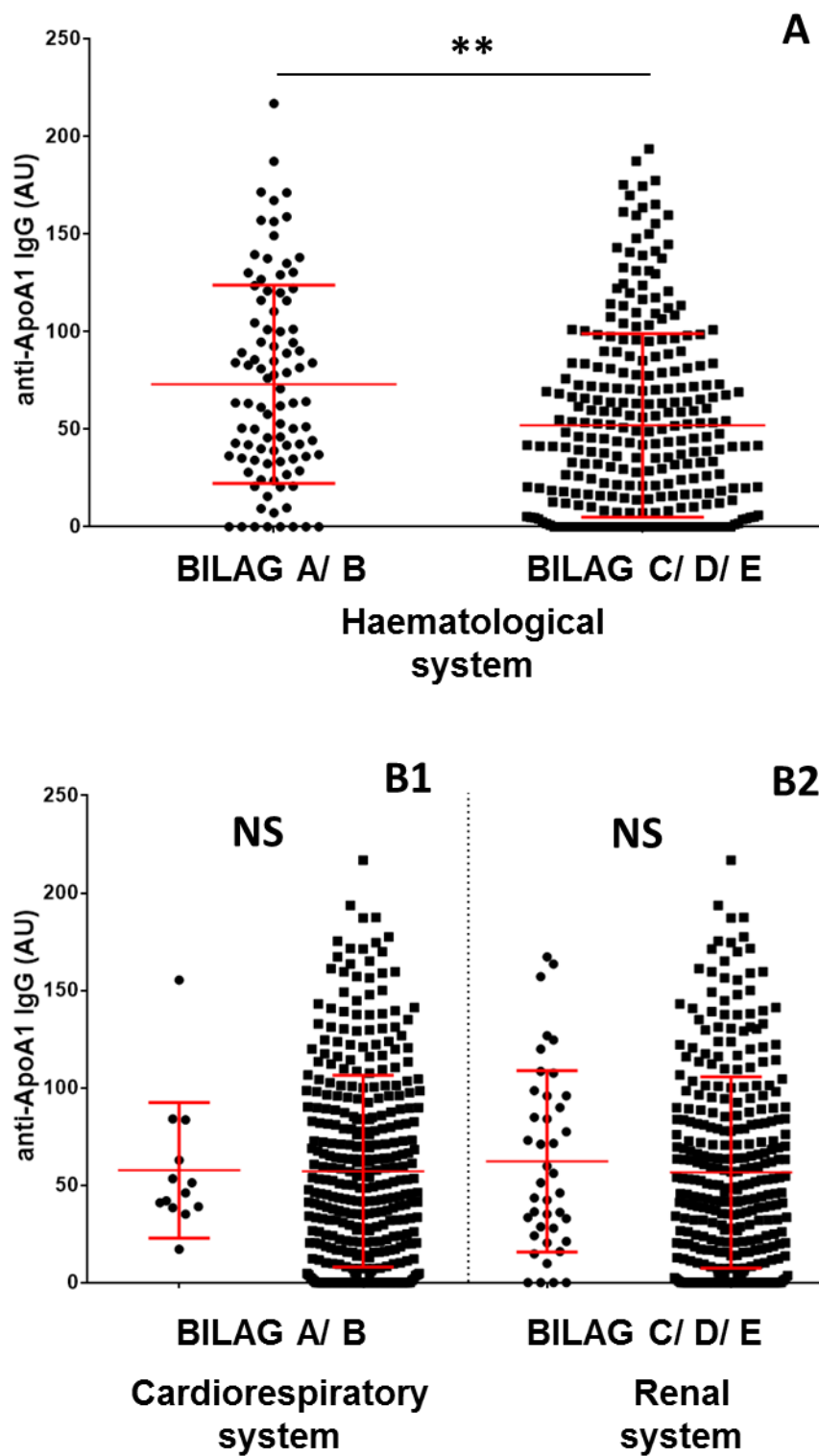


Figure 21: Comparison of anti-ApoA1 IgG levels between BILAG scores of A/ B or C/ D/ E in the haematological (A), cardiorespiratory (B1) and renal (B2) systems.

(** $p < 0.01$)

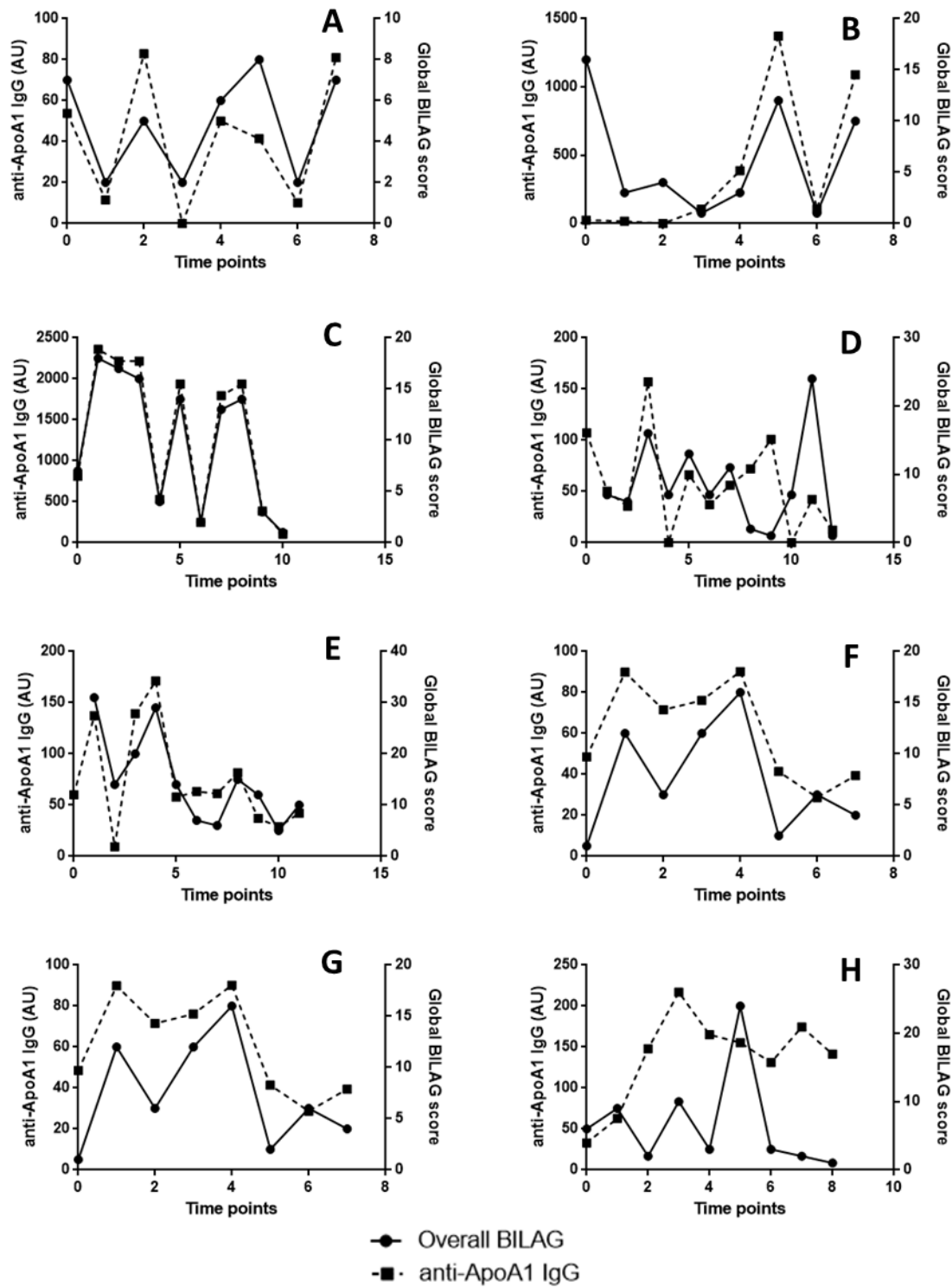


Figure 22: Longitudinal variation in anti-ApoA1 IgG levels and global BILAG score: longitudinal variation of anti-apoA1 levels and disease activity for eight different patients with SLE is represented. Anti-ApoA1 IgG levels parallel global disease activity in many (A-F) but not all (G-H) patients.

When looking at the influence of treatment on Anti-ApoA1 levels, I considered whether or not the patients were taking either HQ or IS, as well as the prednisolone dose they were taking at the time the sample was obtained.

Interestingly, I found that in the samples from patients who were taking HQ had anti-ApoA1 IgG levels 30% lower than samples from those who were not ($p= 0.003$). Similarly, patients who were on low dose steroids ($\leq 7.5\text{mg}$ prednisolone daily) at the time the sample was taken had almost 40% lower anti-ApoA1 levels than those taking higher doses ($p= 0.002$). Figure 17 (A1 and A2) shows these two findings.

No association between was found between anti-ApoA1 levels and treatment with IS at the time that the sample was taken.

To investigate the association between treatment and anti-ApoA1 levels further, I compared the overall BILAG scores between patients taking HQ and low dose steroids with those not on HQ and moderate-high prednisolone dose (Figure 23 – A1 and A 2 respectively).

I found that samples from patients on a lower prednisolone dose also had significantly lower disease activity ($p= 0.017$) which could potentially be a confounding factor. However, no difference in disease activity was found between patients taking HQ compared to those who were not, suggesting the effect of HQ could be independent of disease activity (Figure 23 - B1 and B2).

To investigate the independent effect of each of the variables found to be significantly associated with anti-ApoA1 IgG levels, multivariable analysis was carried out. These findings are summarized in Table 11.

No collinearity was observed between variables and the only variables which remained independently associated with higher anti-ApoA1 IgG levels were negativity for anti-La and anti-Sm, haematological disease activity and higher prednisolone dose.

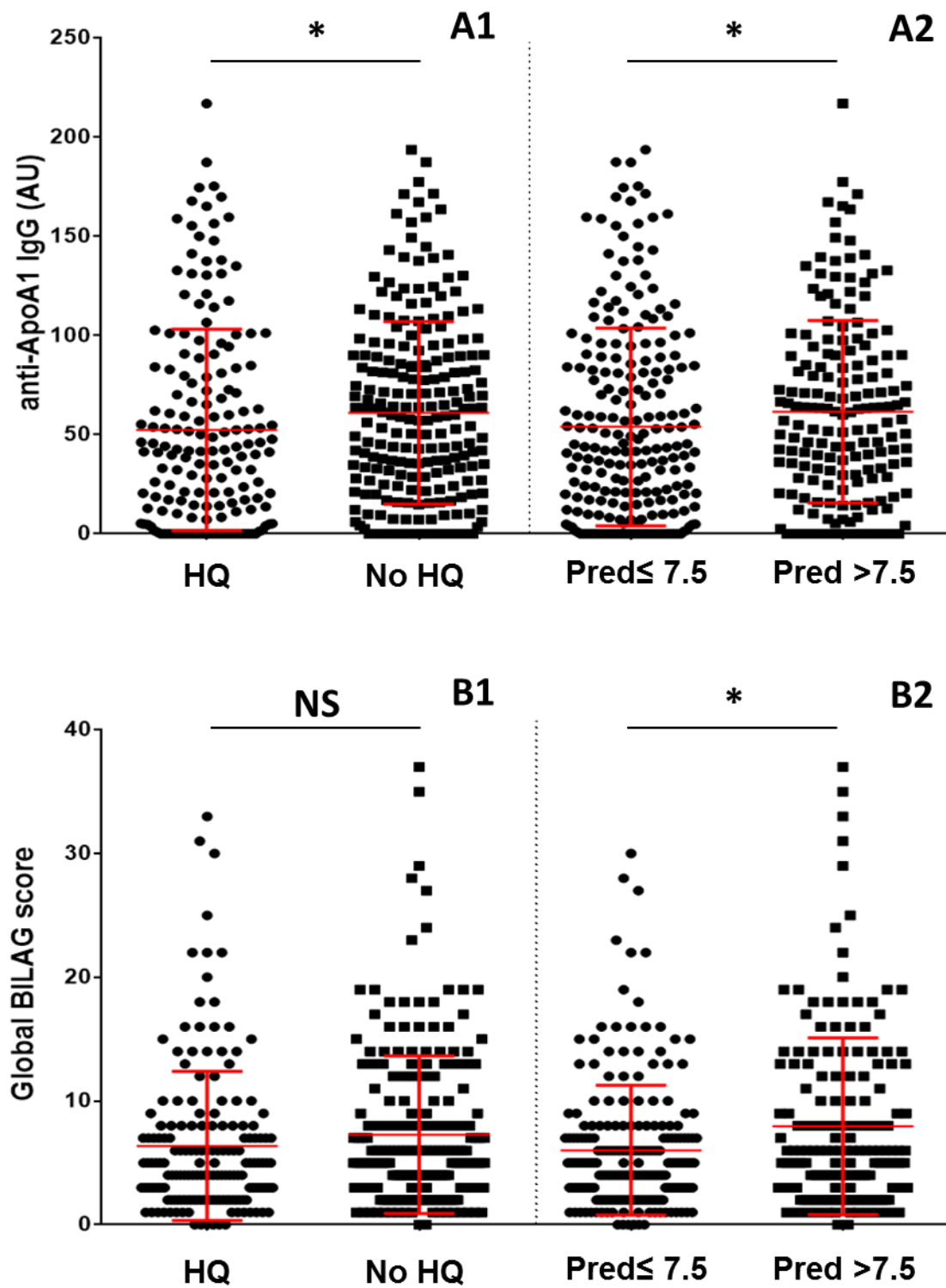


Figure 23: Anti-apoA1 antibody levels and treatment: comparison of anti-ApoA1 levels between patients treated with or without HQ (A1) and low vs. high prednisolone dose (A2) as well as comparison between the global BILAG score in each of the 4 groups considered (B1 and B2). (* $p < 0.05$)

Variable	Category	Ratio (95% CI)	P-value
Anti-La	No	1	0.006
	Yes	0.58 (0.39, 0.86)	
Anti-Sm	No	1	0.001
	Yes	0.58 (0.43, 0.80)	
Haematological system	A, B	1	<0.001
	C, D, E	0.66 (0.53, 0.83)	
Prednisolone dose	≤ 7.5mg/day	1	0.008
	> 7.5mg/day	1.34 (1.08, 1.66)	

Table 12: Multivariable analysis of factors associated with anti-ApoA1 IgG antibodies levels in a longitudinal cohort of patients with SLE.

4.3.1.2: Cross-sectional assessment of anti-ApoA1 IgG levels

Anti-apoA1 IgG levels were tested in two separate cross sectional cohorts: the early SLE group and the vascular US group.

- **Early SLE cohort**

Considering the patients' immunological profile, 26 were ENA positive: 21 were anti-Ro positive, 6 La-positive, 15 anti-RNP positive and 11 anti-Sm positive. Furthermore, during the follow-up period, 59% ($n=29$) had at least one positive anti-dsDNA measurement, 65% ($n=32$) had low C3 on at least one occasion and over 90% ($n=46$) had a flare defined clinically by the classic BILAG score. All the eight systems of the classic BILAG were active at some point in the study.

Based on the positivity cut-off previously described (46.7 AU), over 27% ($n=134$) of the 499 patients included in this cohort were found to be positive for anti-ApoA1 IgG in the first sample obtained within two years of SLE diagnosis (median 21.2; IQR 9.4-52.5). There was no correlation between the order of acquisition of samples and anti-apoA1 IgG level (Spearman correlation coefficient -0.115) suggesting that the storage time does not affect anti-apoA1 IgG levels. As reported above regarding the longitudinal group, no significant associations between anti-apoA1 IgG levels and age, gender or ethnicity were found. The mean anti-apoA1 IgG levels were significantly higher in patients with SLE (median 21.4 AU; IQR 11.2-52.9) compared with healthy controls (8.0 AU; IQR 5.9-10.7) ($p<0.05$).

Considering serological variables, I found that during the course of their follow-up, over 2/3 ($n=326$) of patients were found to be anti-dsDNA positive while anti-cardiolipin antibodies were found to be present in 24.0% ($n=124$). One quarter of the patients were found to be rheumatoid factor positive and over half were ENA positive (14.9% anti-Sm, 27.8% anti-RNP, 37.5% anti-Ro and 13.9% anti-La).

Significantly higher anti-ApoA1 IgG levels at the time of SLE diagnosis were found in patients who were or became anti-dsDNA positive (60.6 AU vs. 34.1 AU; $p=0.0025$). A similar finding was observed for those who developed anti-cardiolipin positivity compared to those who did not (80.1 AU vs. 41.6 AU; $p=0.0062$). Both these findings are depicted in Figure 24. No further associations between anti-ApoA1 IgG levels and other autoantibodies were found.

When considering SLE-associated damage defined according to the SLICC Damage index, I found that positivity for anti-apoA1 IgG did not predict onset of damage during follow-up. As shown on Table 13, no statistically significant differences in damage scores at 5, 10, 15 and

20 years were observed between the different quartiles of anti-apoA1 IgG levels early in the course of SLE.

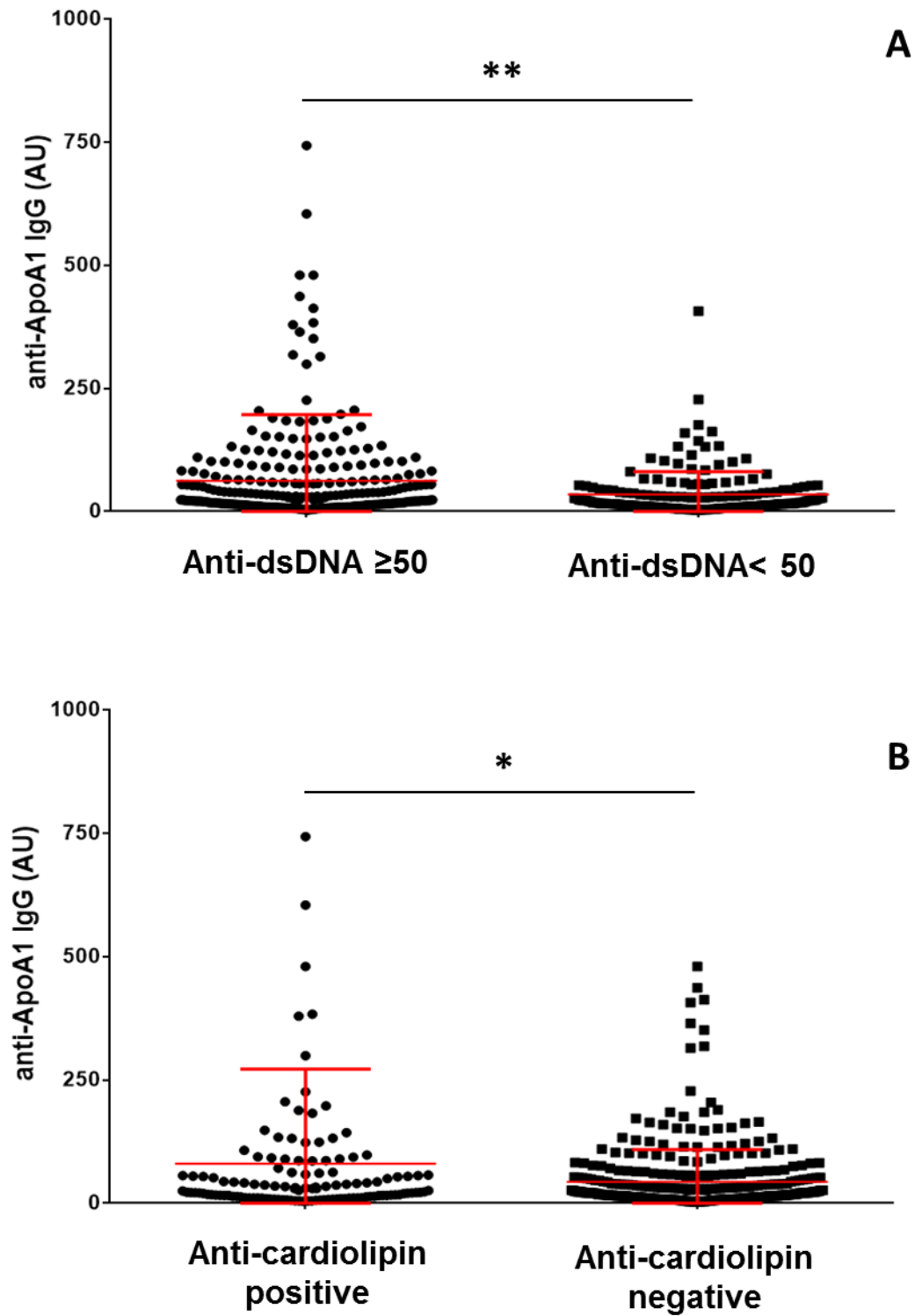


Figure 24: Early anti-apoA1, anti-dsDNA and anti-cardiolipin antibody levels: differences in anti-ApoA1 IgG levels measured in early disease sera samples between patients found to be anti-dsDNA positive or negative (**A**) or anti-cardiolipin positive or negative (**B**).

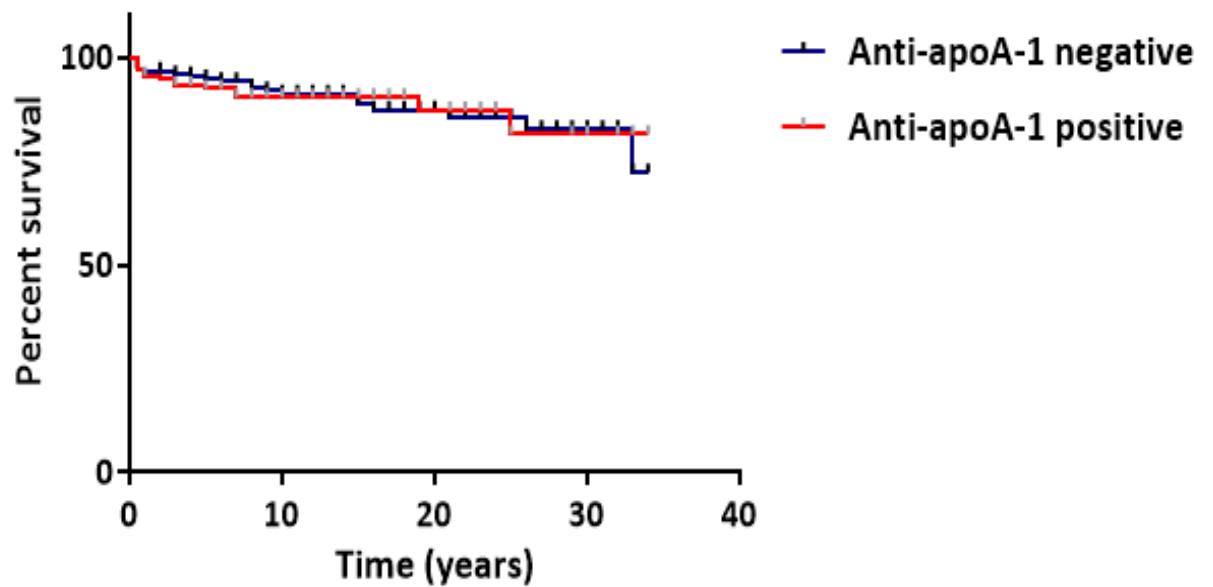
(* $p < 0.05$; ** $p < 0.01$)

Damage score	Lowest quartile	Second quartile	Third quartile	Highest quartile	P value
At 1 year (n=236)	0.14	0.13	0.10	0.08	>0.05
At 5 years (n=236)	0.63	0.43	0.45	0.42	>0.05
At 10 years (n=209)	1.06	0.60	0.96	0.65	>0.05
At 15 years (n=131)	1.23	0.81	1.33	0.97	>0.05
At 20 years (n=69)	1.33	1.06	1.88	1.00	>0.05

Table 13: Mean damage scores according to the SLICC Damage Index over a 20 year follow up stratified according to quartiles of anti-ApoA1 IgG levels in early disease sera samples.

During the course of follow-up, 40 patients (8.0%) suffered a CVD event (ischaemic heart disease confirmed either by raised cardiac enzymes and/ or ECG changes suggestive of MI and/ or by angiography in NSTEMI; or ischaemic stroke confirmed by imaging). Overall, the prevalence of CVD events was similar between anti-ApoA1 positive and negative patients (6.7% vs 8.5%; $p>0.05$).

In order to further explore potential differences in CVD-related mortality and/or morbidity, the 499 patients were divided into quartiles based on their anti-apoA1 IgG levels. I found that patients in the upper quartile were just as likely to have a CVD event compared with the lower quartile (prevalence 6.5% vs. 10.6%; $p>0.05$). A Kaplan-Meier Survival curve showing the proportion of patients CVD-free in the anti-apoA1 positive and negative groups at all-time points up to 34 years follow-up showed no statistically significant difference between the two groups ($p= 0.89$ by log-rank test) as shown on Graph 7.



Graph 7: CVD-free survival analysis: A Kaplan-Meier Survival curve shows no statistically significant differences in the proportion of patients CVD-free in the anti-apoA1 positive and negative at all-time points up to 34 years follow-up.

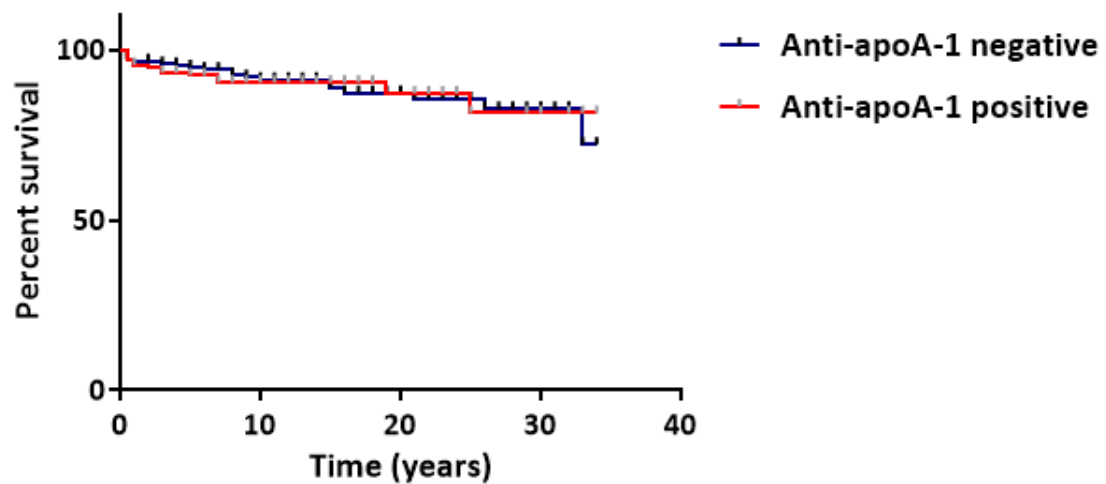
Considering the overall mortality, 66 of the 499 subjects died during the course of follow up and over 70% of these died before the age of 60 ($n=48$). When looking at the anti-ApoA1 IgG levels of these patients, those who tested positive in their early sample appeared to be more likely to have died than those who tested negative (17.1% vs. 12.4% mortality respectively), but this difference did not reach statistical significance ($p=0.11$).

I then looked at the different anti-ApoA1 quartiles and found that there were 39 deaths in the upper quartile compared to 27 in the lower quartile ($p=0.07$).

This pattern was more striking when looking at early mortality, i.e. deaths occurring below the age of 60. In this instance, I stratified patients based on their serum anti-ApoA1 IgG levels into upper (>21.2 AU) and lower halves (≤ 21.2 AU) than quartiles to include all the patients and found that there significantly more deaths in the upper compared with the lower half (31 vs. 17; $p=0.023$). However, as shown on Graph 8, a Kaplan-Meier survival curve showing the percentage survival of patients in the anti-apoA1 positive and negative groups at all-time points up to 34 years follow-up showed no statistically significant differences between groups.

Furthermore, there was no evidence to support the idea that early deaths in patients with higher anti-apoA1 IgG were due to CVD. Table 14 shows the causes of death for patients who died before or after 60 years of age and in summary no differences between these two groups were found.

Of the six patients who died from CVD under the age of 60, only one was anti-apoA1 IgG positive at the time of diagnosis.



Graph 8: Survival analysis: a Kaplan-Meier survival curve showing the percentage survival of patients in the anti-apoA1 positive and negative groups at all-time points up to 34 years follow-up showed no statistically significant differences between groups.

Cause of death	Death< 60 years (n=48)	%	Deaths ≥60 years (n=18)	%
CVD-related events	6	13	3	17
Infection	15	31	3	17
Cancer	8	17	8	44
Renal	4	8	1	6
Other	15	31	3	17

Table 14: Causes of death in patients before and after the age of sixty years showing no significant differences between the two groups.

Vascular US cohort

The findings of the vascular US group were similar to those reported for the longitudinal and early SLE cohorts and will be explored with further detail on section 4.3 Vascular Ultrasound.

Briefly, the median anti-apoA1 level at the time of the scan was 66.7 AU (IQR 31.1-181.0) and two thirds of the patients (n= 66) were found to have levels greater than the positivity cut-off previously defined (46.7AU). Age at scan and C3 levels were found to have a significant negative correlation with anti-ApoA1 IgG levels (r^2 -0.23 and -0.25; p 0.03 and 0.012 respectively). As previously described for the early disease cohort, an association between early anti-ApoA1 IgG levels and anti-dsDNA was observed (r^2 0.3; p= 0.0006). No further statistically significant associations with auto-antibody profile were found.

A significant positive correlation between early anti-ApoA1 levels and mean CCA IMT was found (r^2 0.24; p=0.021) but no further associations between anti-ApoA1 IgG levels and either total plaque area (TpA) or total plaque thickness (TpT) were observed. Similarly, anti-ApoA1 levels did not appear to influence plaque texture assessed by the GSM value.

4.3.2: Anti-ApoA1 IgM antibodies

A cross-sectional assessment of anti-apoA1 IgM levels was done using sera belonging to the patients included in the vascular US cohort ($n= 100$).

Patients with SLE had significantly higher levels than HC (median 17.2 vs 7.8; IQR 3.9-107.5 vs 4.7-13.0) as shown on Graph 9. The positivity cut-off was defined as the 97.5th percentile of the 100 HC tested (167.2 AU) and under one third of the patients ($n= 22$) was found to be positive.

The relevant findings regarding the factors influencing the anti-ApoA1 IgM levels are summarized in Table 15.

No associations between general demographic variables and anti-ApoA1 IgM levels were found. Although a significant correlation with total serum IgM levels and anti-ApoA1 IgM was noted, no further correlations with general laboratorial findings were established, namely with lipid profile.

With regards to the auto-antibody profile, patients who were anti-RNP positive had significantly lower anti-ApoA1 IgM levels ($p= 0.01$) but no further significant associations emerged.

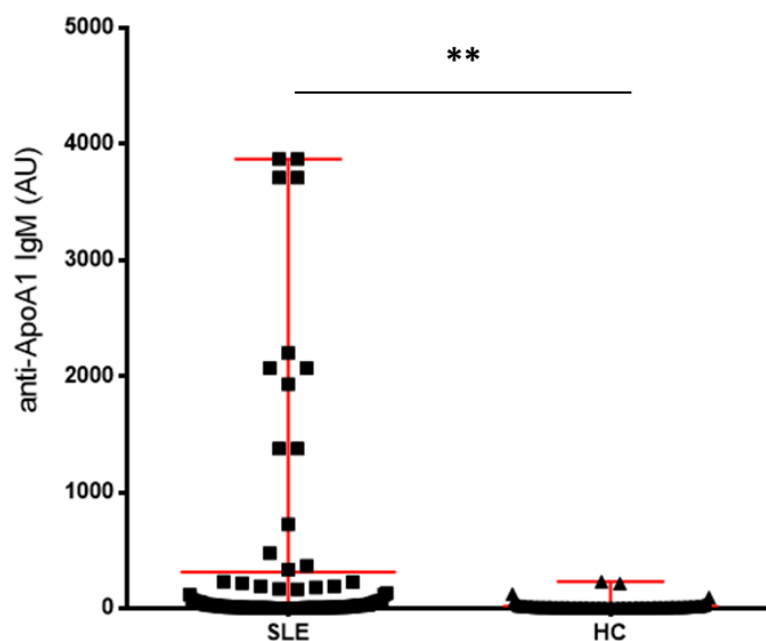
A positive correlation between anti-ApoA1 IgM levels and GSM was found but no statistically significant associations with other US-related variables were observed.

Disease activity appeared to correlate with anti-ApoA1 IgM levels, specifically the global BILAG score during the first year after diagnosis. A non-significant trend was found between sustained disease activity and anti-ApoA1 IgM levels: patients with higher levels of anti-ApoA1 IgM appear to be more likely to have persistently low activity while the inverse was found for patients who were persistently active. No significant associations with other clinical and serological markers of disease activity were observed.

Patients who had undergone treatment with B-cell depletion, i.e. rituximab +/- cyclophosphamide according to the Rheumatology department protocol at UCLH ($n= 31$), had significantly lower anti-ApoA1 IgM levels. However, they were also found to have significantly lower total serum IgM levels ($r^2 -0.40$; $p< 0.0001$) which could potentially be a confounding factor. In addition, patients who were treated with B-cell depletion had significantly more active disease, with a higher global BILAG score at the time of scan ($p=0.04$) and higher mean global BILAG score during follow-up ($p< 0.0001$). Moreover, these patients had a greater number of visits with ≥ 1 A flare and/or ≥ 2 B flares in at least one

organ system ($p= 0.0007$ and 0.003 respectively) and their mean and cumulative global BILAG score during the first year of follow up ($p= 0.0004$ and 0.0002 respectively) was also higher. Figure 25 illustrates these findings.

Finally, 16 patients were positive for both anti-apoA1 IgG and IgM. Over 70% of the anti-ApoA1 IgM positive patients were also anti-apoA1 IgG positive (16/22), while only less than a quarter of patients found to be anti-ApoA1 IgG positive were also anti-apoA1 IgM positive. Combined positivity between early anti-ApoA1 levels and either anti-apoA1 IgG or IgM was found in a minority of patients (12/66 and 7/22 respectively. However, as previously stated, when considering the power of correlation, these findings did not reach statistical significance (Spearman's correlation; CI 95%).



Graph 9: Comparison between anti-ApoA1 IgM levels between patients with SLE and healthy controls (HC).

(** $p < 0.01$)

Variable	r^2 (95% CI)	p-value
RNP positivity	-0.26 (0.1 – 0.47)	0.01
Disease activity		
Mean global BILAG score during the 1 st year after SLE diagnosis	-0.21 (0.1 – 1.2)	0.01
Cumulative global BILAG during the 1 st year after SLE diagnosis	-0.22 (0.2 – 1.0)	0.04
Mean global BILAG score/ year	-0.23 (0.5 – 1.2)	0.03
Serum IgM (g/L)	0.44	<0.0001
B-cell depletion	-0.35	0.003

Table 15: Factors significantly correlated with anti-ApoA1 IgM (Spearman's correlation; CI 95%).

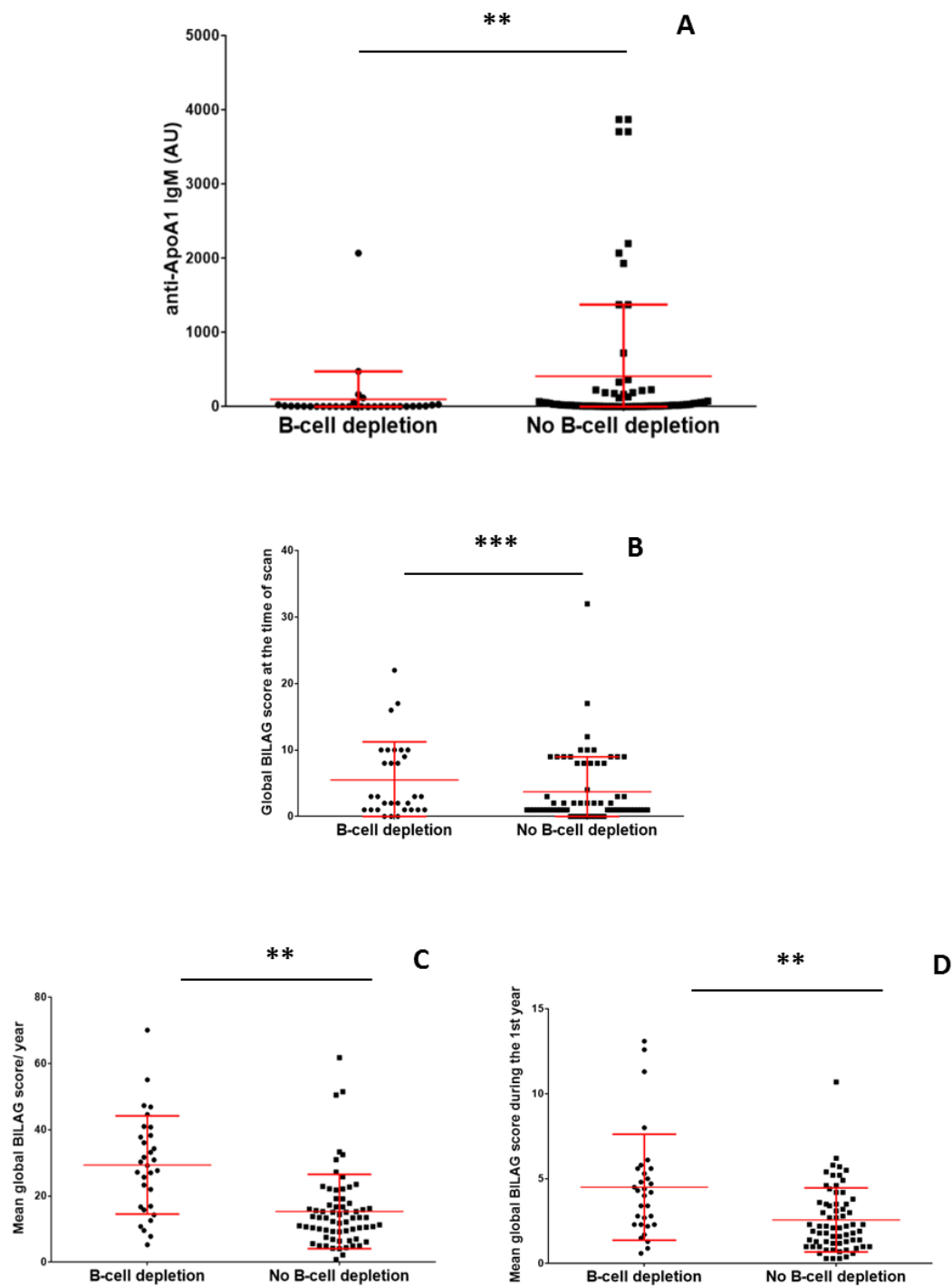


Figure 25: Anti-apoA1 IgM levels and B-cell depletion: comparison between anti-ApoA1 IgM levels between patients treated with B-cell depletion and those who did not receive this treatment (A) and comparison of disease activity between these two groups at the time of the scan (B), during follow up (C) and over the first year of follow up (D).

(** $p < 0.001$; ** $p < 0.01$)

4.3.3: Anti-HDL IgG antibodies

Anti-HDL IgG antibodies were tested cross-sectionally in the vascular US group.

Patients with SLE had significantly higher anti-HDL levels than HC (median 7.7 vs 1.9; IQR 0.0-24.9 vs 0.0-8.4) as shown on Graph 10. The upper limit of normal was defined as the 97.5th percentile based on the HC measurements as 22.5 AU and 27 patients were found to be positive.

Although a significant correlation between anti-HDL levels and age at scan was found (r^2 0.22; $p=0.04$ CI 95%), no associations with ethnicity and auto-antibody profile, including anti-ENA were noted. Although the majority of patients found to be anti-HDL positive were also anti-ApoA1 IgG positive (18/27), the reverse was not observed as less than a third of anti-ApoA1 positive patients were also anti-HDL positive (18/66). Moreover, over one third of anti-HDL positive patients were also found to be anti-apoA1 IgG positive on their early disease sample (10/27). However, only a minority of patients had combined positivity for both anti-HDL and anti-ApoA1 IgG (3/27).

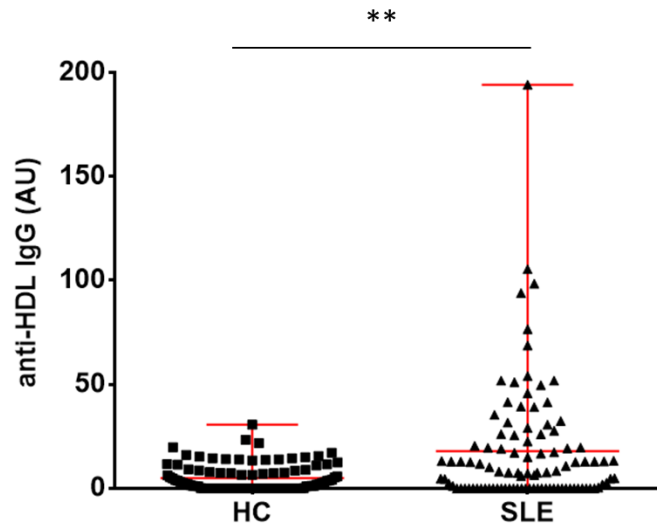
A statistically significant negative correlation between anti-HDL and total ApoA1 levels was observed (r^2 -0.21; $p=0.03$; CI 95%). This finding was further illustrated when comparing total ApoA1 levels between the upper quartile (anti-HDL >24.9 AU) and the lower quartile (anti-HDL \leq 0.0 AU) of anti-HDL as shown on Figure 26-A.

However, no significant associations between anti-HDL and lipid profile, namely with HDL levels was noted even when comparing the upper and lower quartiles of anti-HDL antibodies (Figure 26-B).

Similarly, disease activity defined either clinically through the BILAG score or serological by anti-dsDNA and C3 was not found to correlate significantly with anti-HDL.

No associations between anti-HDL and nitrated protein levels, nitrated ApoA1 or NN, were found.

Anti-HDL levels also correlated with vascular US findings, in particular with TpA and TpT and these findings will be further explored in section 4.3 Vascular Ultrasound.



Graph 10: Comparison of anti-HDL IgG levels between patients with SLE and HC ($p < 0.01$).**

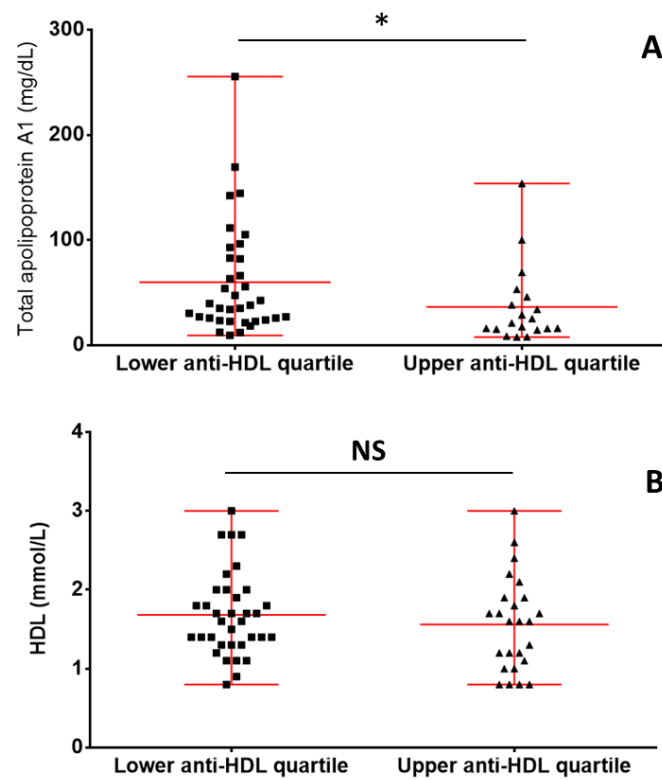


Figure 26: Comparison of total serum apolipoprotein A1 (A) and HDL levels (B) between the upper and lower quartiles of anti-HDL IgG (* $p < 0.05$; NS: non-significant).

4.3.4: Discussion

Comparable to what has been previously reported (Dinu, Merrill et al. 1998, Delgado Alves, Ames et al. 2002, Alves and Ames 2003, Delgado Alves, Kumar et al. 2003, Batuca, Ames et al. 2007, Batuca, Ames et al. 2009, O'Neill, Giles et al. 2010); I found that anti-ApoA1 levels were significantly higher in SLE compared to healthy controls in all the groups studied. Interestingly, 27% of 499 patients were positive for anti-apoA1 IgG very early in the disease course. Arbuckle et al showed that many autoantibodies are present in patients with SLE even in stored samples taken before diagnosis of SLE (Arbuckle, McClain et al. 2003), but results of anti-apoA1 levels measured in early disease samples have not been published to date. The association between autoantibody levels and disease activity is not a new concept and other papers have also shown associations between serological markers and activity in particular systems; anti-nucleosome antibodies and renal lupus (Manson, Ma et al. 2009), interleukin-6 and haematological lupus (Ripley, Goncalves et al. 2005) and nitrated nucleosomes and vasculitis (Croca, Bassett et al. 2014). Nonetheless, none of these biomarkers has substituted C3 and anti-dsDNA as the gold standard for assessing lupus activity.

O'Neill et al showed that anti-apoA1 IgG levels were higher in patients with persistently active disease compared with those with quiescent disease and that they rose at times of disease flare (O'Neill, Giles et al. 2010). This study was limited by its cross-sectional nature as did not study serial samples taken over long periods, thus preventing the use of multivariable analysis and elimination of potential confounding factors. I tested multiple samples taken from 49 patients longitudinally and univariate analysis suggested an association of anti-apoA1 levels with disease activity defined both clinically through the BILAG score and serologically through C3 and anti-dsDNA levels. Patients who had a haematology score of either A or B at the time of the sample had significantly higher levels of anti-ApoA1 than those with scores of C, D or E. Similarly, patients with an overall BILAG score greater than 5 at the time of the sample had greater anti-ApoA1 levels than those with a lower score. HQ appeared to have a similar effect, as samples taken at a time when the patients were taking HQ had significantly lower anti-ApoA1 levels. However, when using multivariate analysis, then only variables whose associations with anti-ApoA1 levels remained statistically significant were the presence of active haematological disease (BILAG score A/B), higher corticosteroid dose (prednisolone >7.5mg daily) and negativity for anti-La and anti-Sm.

The differences between the univariate and multivariate analysis regarding the associations between steroid dose, HQ and disease activity can be explained by the presence of

confounding factors, such as disease activity, given that the treatment regimen, namely the steroid dose is chiefly determined by the clinical scenario. However, the power of multivariate analysis also lies in the number of samples available and how many variables are considered and it can be argued that it may disregard potentially important associations, particularly if two variables are closely related. In fact, when I compared the disease activity between patients on ≤ 7.5 mg prednisolone daily and those on higher doses, I found that the former also had lower disease activity. Therefore, it appears that the association between anti-ApoA1 and steroid dose may be due to the disease activity at the time of the sample rather than to the steroid dose *per se*. However, the same was not true for HQ. When I compared the disease activity between samples taken when the patients were on HQ vs. those when the patients weren't on HQ, I found no differences. This may signify that the correlation between HQ and lower anti-ApoA1 levels is not due to disease activity, but may in fact depend on the effect of HQ.

Although the association between anti-ApoA1 antibodies and treatment with HQ was not supported by the multivariate analysis, the beneficial role of HQ on the lipid profile has been suggested in RA. Wallace et al (Wallace, Metzger et al. 1990) reported a 15-20% decrease in TG, t-cholesterol and LDL in patients with RA who were taking HQ, suggesting that it could potentially reverse the deleterious effects of steroids. Munro et al ((Munro, Morrison et al. 1997) reported further benefits of HQ use in HDL, showing that after 6 months of treatment, total HDL and the HDL/ t-cholesterol ratio were significantly increased, with median HDL levels raising by 15%. To place these results in context, it has been shown that a 10% fall in serum t-cholesterol leads to a 10% decrease in death from MI and of 21% of non-fatal ACS (Law, Wald et al. 1994). Other measures to reduce cholesterol have variable effects in the lipid profile: lipid lowering diet can lead to a 2-13% reduction in total cholesterol (Ramsay, Yeo et al. 1991), while statins usually elicit a 20-30% reduction in total cholesterol and 15% raise in HDL (Shepherd, Cobbe et al. 1995). More recently, a study by Morris et al (Morris 2011) shows that the use of HQ is independently associated with lower LDL, t-cholesterol, LDL/HDL and t-cholesterol/ HDL ration in a cohort of 706 patients with RA. A non-significant trend towards with HDL and lower TG levels in patients taking HQ was also noted.

Although it is considered to be a disease modifying drug (DMARD) in RA, HQ has a relatively weak anti-inflammatory effect and is therefore unlikely that its impact on the lipid profile is due to a better control of systemic inflammation. *In vivo* studies showed that chloroquine, which is structurally similar to HQ, inhibits cholesterol synthesis in rat hepatocytes (Beynen, van der Molen et al. 1981). It has been suggested that this effect may

be due to the fact that HQ lowers the lysosomal pH which could in turn inhibit the proteolysis of internalized cholesterol esters (Beynen, van der Molen et al. 1981). Even if the use of HQ is not associated with reduced anti-ApoA1 levels all the available evidence supports its use in SLE not only to treat symptoms directly related with the disease but also to potentially provide some degree of cardiovascular protection.

The atheroprotective role of ApoA1 has been extensively reported (Hahn 2010, O'Neill, Giles et al. 2010, Teixeira, Cutler et al. 2012). The occurrence of dysfunctional HDL in inflammatory diseases such as SLE has also been suggested as a potentially important factor in the increased CVD risk observed in these patients (Hahn, Grossman et al. 2007). Several studies suggest that one of the mechanisms through which HDL function is impaired may be the presence of anti-ApoA1 (Batuca, Ames et al. 2007, Batuca, Ames et al. 2009, O'Neill, Giles et al. 2010, Vuilleumier, Bratt et al. 2010). Previous studies confirmed the presence of IgG anti-apoA1 antibodies in patients with SLE, indicated an association with higher disease activity and suggested a possible link with the development of atherosclerosis (Shoenfeld, Szyper-Kravitz et al. 2007, Batuca, Ames et al. 2009, O'Neill, Giles et al. 2010, Radwan, El-Lebedy et al. 2013). However, to establish unequivocally a causal effect, long-term follow up is required, including vascular scanning data and multiple serial samples from patients with fluctuating disease activity. No previous study has fulfilled all these requirements but in my thesis I have tried to do so by studying three distinct but overlapping patient groups: longitudinal, early SLE and vascular US.

I found no association between anti-apoA1 IgG positivity early in disease and development of CVD later. The numbers of patients with CVD were relatively small (42/518), but I also found no association between anti-apoA1 and the presence of carotid or femoral plaque or increased CCA IMT in the 100 patients scanned. Moreover, the presence of anti-ApoA1 antibodies did not appear to influence plaque texture assessed by GSM. Thus no convincing link between anti-apoA1 and atherosclerosis or CVD in patients with SLE has emerged. This agrees with the findings reported by Radwan et al in Egyptian patients with SLE (Radwan, El-Lebedy et al. 2013) but contrasts with those of Vuilleumier et al , who found that, in 133 Swiss patients with RA followed for a median of 9 years, baseline IgG anti-apoA1 positivity was strongly associated with risk of developing a major cardiovascular event (Hazard Ratio 4.7, 95% CI 1.9-11.2) even after adjusting for standard CVD risk factors in multivariable analysis (Vuilleumier, Reber et al. 2004). However, this RA cohort was older (mean age 65 years) contained more males (30%) and had a higher rate of CVD events compared to our group or other SLE groups reported in the literature (Shoenfeld, Szyper-Kravitz et al. 2007, Batuca, Ames et al. 2009, O'Neill, Giles et al. 2010, Radwan, El-Lebedy et al. 2013). It may

well be that longer-term studies will be needed in SLE than in RA to identify predictive effects of factors such as autoantibodies on CVD risk. Notably, in a large inception cohort study, 1249 patients with SLE were followed for a median of 8 years and only older age and male gender were identified as CVD risk factors in multivariable analysis (Urowitz, Ibanez et al. 2007, Urowitz, Gladman et al. 2010).

Although no associations between anti-ApoA1 and CVD emerged, I found a statistically significant association between anti-apoA1 positivity early in disease and increased risk of dying before the age of 60. The reason for this association is unexplained. Development of damage (due to disease activity or therapy) is a powerful risk factor for mortality (Rahman, Gladman et al. 2001) in SLE but I found no association between anti-apoA1 IgG levels and SLICC-DI score over a 10-year follow-up period on over 200 patients. However, many of the patients who died before the age of 60 were not represented amongst these 200, their early death precluding long-term follow-up.

Regarding anti-ApoA1 IgM antibodies, I decide to measure their levels in the vascular US cohort due to the potential protective role attributed to IgM antibodies not only in autoimmunity, but also in atherosclerosis. Manson et al summarized in a comprehensive review the protective role played by natural IgM in several processes traditionally implied in the development of autoimmunity (Manson, Mauri et al. 2005). The potentially protective role of IgM may be attributed to its low affinity, predominantly intravascular distribution and lack of interaction with Fc-receptors in phagocytic cells (Virella 1993).

Ajeganova et al tested the sera of 215 patients with RA and found that those with higher anti-phosphorylcholine IgM antibodies at baseline were more likely to remain in remission regardless of the treatment chosen (rituximab versus anti-TNF agent). The authors suggest that the presence of low levels of this specific type of IgM antibodies may identify an immunological profile which predicts inferior therapy response (Ajeganova, Fiskesund et al. 2011). Stoehr et al also described how the production of self-reactive IgM induced by TLR9-expressing peritoneal B1 cells in lupus-prone mice led to reduced Th17 cell development and consequently abrogated severe autoimmunity (Stoehr, Schoen et al. 2011). The protective role of IgM-producing serosal B1-cells appears to also extend to atherosclerosis as reported by Kyaw et al who review their potentially atheroprotective role (Kyaw, Tipping et al. 2012).

Considering the immunological basis for atherosclerosis, several studies have reported on the potentially atheroprotective role for IgM. Chronic kidney disease has a strong correlation with CVD and CVD-related risk factors such as diabetes and several studies have been

developed to assess the role of IgM antibodies. Virella et al measured IgG and IgM antibodies directed against oxidized LDL in a cohort of patients with type I diabetes (n=36), a group notoriously recognized as having increased CVD risk. They found a predominance of IgG antibodies in diabetic patients compared with HC and this difference was enhanced when considering patients who had diabetic nephropathy. However the data reported were not enough to support the protective role of IgM antibodies (Virella, Carter et al. 2008). Similarly, Carrero et al reported that low level of natural IgM directed against the exposed phosphorylcholine in inflammatory phospholipids is an independent predictor of death among patients who are under haemodialysis (Carrero, Hua et al. 2009). Yilmaz et al report how HIV-positive patients have significantly lower IgM and higher IgG antibodies directed against oxidized LDL and argued that this may be a contributing factor to the excess CVD risk found among these patients (Yilmaz, Jennbacken et al. 2014).

Interestingly, Karvonen et al described a negative correlation between IgM antibodies directed against oxidized LDL and carotid IMT (Karvonen, Paivansalo et al. 2003). The opposite though was reported by Fredrikson et al who found a more rapid IMT progression of carotid disease in the presence of IgM directed against apolipoprotein B-100 (Fredrikson, Hedblad et al. 2007). Animal studies conducted by Cesena et al showed that in hypercholesterolemic apoE deficient mice, treatment with polyclonal IgM reduced aortic atherosclerosis (Cesena, Dimayuga et al. 2012). Lewis et al also described their findings on the influence of natural IgM antibodies on atherosclerosis. They selected serum from IgM deficient mice and found that compared with normal controls these had larger aortic atherosclerotic plaque, more complex crystal formation and increased smooth muscle cell content. Moreover, this effect appeared to be at least partly independent of C1q (Lewis, Malik et al. 2009).

I found a weak positive correlation between anti-ApoA1 IgM levels and GSM, but no other associations with US findings was observed. Higher GSM is associated with increased connective tissue deposition in the plaque and as discussed in the introduction this may signify greater plaque stability. However, the mechanism through which anti-ApoA1 IgM antibodies could lead to these changes has not been previously discussed in the literature. I did not find any statistically significant associations between anti-ApoA1 IgM levels and disease activity or autoantibody profile. Patients who were treated with rituximab did have lower anti-ApoA1 IgM levels but they also had lower total serum IgM levels and therefore I did not think this to be a relevant finding.

To date, there are no papers reporting on the measurement of anti-Apoa1 IgM antibodies or their relation with either disease activity or markers of atherosclerosis.

4.4: Vascular Ultrasound

4.4.1: Overview of vascular ultrasound results

4.4.1.1: Cohort characterization

103 patients were scanned using the protocol previously described. However, three of the patients scanned were later felt not to fulfil the revised 1982 ACR criteria for SLE (Hochberg 1997) and were therefore excluded from the analysis ($n= 100$).

95% of the patients were women and the overall mean age was 45.2 years (SD 12.4; range 20-66). The mean age at SLE diagnosis was 29.2 (SD 10.9; range 8-56) and on average patients had been under follow up in for 16 years (SD 10.0; range 2-46).

56 patients were Caucasian, 25 were of Afro-Caribbean-origin, 11 were South Asian and 8 patients had other ethnic backgrounds (Chinese or mixed race).

Overall, patients had a broad spectrum of organ involvement, most commonly skin, joints and kidney. Biopsy proven lupus nephritis was present in 37 patients. A few patients had had vasculitis and/or serositis during the course of their disease while definite neurological involvement was exceedingly rare ($n= 1$). Less than one third of the patients ($n= 26$) had a formal diagnosis of hypertension and a very small minority had diabetes ($n=2$). Although only 10 patients were smokers at the time of the scan, 17 had been smokers at some point in their life.

Data on each individual patient's general laboratory biochemistry assessment, including renal function tests, albumin levels, lipid profile and homocysteine were obtained from the UCH associated laboratory. Further data on auto-antibody profile were also collected. For each patient, BP measurements at the time of the scan were taken as previously described.

Most of these patients had normal BP although sBP was raised in 34 patients; only 22 had sBP greater than 130mmHg.

For the majority of patients, renal function was found to be normal, with serum creatinine levels above the upper limit of normal for only 11 patients. Nonetheless, urine protein:creatinine ratio was found to be raised in almost a quarter of patients ($n= 24$) although serum albumin levels were reduced in only 6 patients.

Treatment with ACE-inhibitors was noted on 35% of patients either for BP control or due to kidney disease and proteinuria.

Despite normal mean values, t-cholesterol, LDL and triglyceride levels were found to be elevated in 43, 46 and 10 patients respectively. Only 8 patients had HDL levels below the lower limit of normal. Treatment with statins was documented in 13 patients.

Regarding vitamin D measurement, over one third of patients (n= 36) were found to have either deficient or insufficient levels and a similar number were or had been on vitamin D supplements recently.

Homocysteine was measured in half the patients and levels were found to be raised compared to what could be expected for the age and gender of the cohort. Treatment with folic acid supplements was only taken by patients being treated with methotrexate.

When considering the auto-antibody profile, 70% of patients were found to be anti-dsDNA positive at some point in their disease course and over half (n= 54) had reduced C3 levels. At the time of scan, close to 40% of patients had either raised anti-dsDNA or reduced C3 levels (n= 38 and 36 respectively).

Close to two thirds of patients (n= 58) were found to be ENA positive and positivity for the different subtypes was as follows: anti-Ro 41%, anti-La 16%, anti-RNP 32% and Sm 17%.

A minority of patients was positive for anti-phospholipid antibody profile (anti-cardiolipin n= 13; anti- β 2GP1 n= 9; lupus anticoagulant n= 12), anti-C1q (n=21), anti-CCP (n=4) or rheumatoid factor (n=7).

Overall, patients were found to have normal serum immunoglobulin levels, although 20% (n= 23) had raised serum IgG levels and almost all of those had high anti-dsDNA levels.

With regards to SLE-related treatment, 65% were taking hydroxychloroquine at the time of scan and 45% were on immunosuppressants. Most commonly, patients were either on azathioprine (n= 17) or MMF (n= 18), while a smaller number was treated with methotrexate (n= 5) or other drugs (n= 5), including tacrolimus and mepacrine).

The mean daily prednisolone dose was 4.6mg but over one third of patients (n= 37) were not taking any steroids at the time of the scan while a minority was taking >7.5mg (n= 14).

Finally, 31 patients had undergone at least one cycle of B-cell depletion during the course of their follow up.

A summary of these findings is presented in Table 16.

Variable	Mean	SD (range)	Normal range
BP (mmHg)			
Systolic	126.3	15.9 (94-168)	120
Diastolic	75.8	10.4 (55-109)	80
Mean	92.6	11 (70-124.7)	100
Renal function tests			
Urea (mmol/L)	5.6	2.9 (2-17.5)	2.5-8
Creatinine (µmol/L)	73.6	31.8 (40-227)	<106
U protein:creatinine (mg/mmol)	50.7	84.0 (4-567)	<45
Albumin (g/L)	43.1	4.9 (19-5)	30-50
Lipid profile			
t-cholesterol (mmol/L)	4.8	1.1 (2.5-7.3)	<5.2
LDL (mmol/L)	2.62	0.9 (0.8-4.8)	<2.0
HDL (mmol/L)	1.7	0.5 (0.4-2.8)	>1.0
Triglycerides (mmol/L)	1.1	1.1 (2.5-7.3)	<2.2
t-cholesterol:HDL	3.1	0.9 (1.6-5.6)	
Homocystein (µmol/L)	14.9	5.6 (8-38)	6-15
Inflammatory markers			
CRP (mg/dL)	2.8	2.7 (0.6-15.5)	<5
ESR (mm/h)	21.1	18.4 (2-86)	<20
Vitamin D (nmol/L)	60.2	33.3 (7-228)	25-137
Anti-dsDNA (IU/L)	96.8	166.7 (1-712)	<50 IU/L
Complement (C3) (mg/dL)	1.0	0.2 (0.49-1.46)	≥0.9g/L
Anti-C1q antibodies			
Positive	21	-	-
Negative	55	-	-
Anti-cardiolipin antibodies			

	Positive	13	-	-
	Negative	82	-	-
Anti-β2 GP1 antibodies				
	Positive	9	-	-
	Negative	20	-	-
Lupus anticoagulant				
	Positive	12	-	-
	Negative	78	-	-
Anti-CCP antibodies				
	Positive	4	-	-
	Negative	36	-	-
Rheumatoid factor				
	Positive	7	-	-
	Negative	65	-	-
Serum immunoglobulins (g/L)		13.3	5.2 (4.62-32.32)	7-16g/L
	IgG	1.1		0.4-2.3g/L
	IgM	2.8	1.0 (0.1-6.6)	
	IgA		1.5 (0.1-9.12)	0.7-4g/L
Treatment				
	Prednisolone (daily dose mg)	4.6	6.1 (0-50)	-
Immunosuppressants (Y/N)		Y = 65 / N = 35		
Hydroxychloroquine (Y/N)		Y = 45 / N = 55		
B-cell depletion (Y/N)		Y = 31 / N = 69		

Table 16: General characterization of the vascular US cohort: blood pressure (BP), general biochemistry laboratory findings, auto-antibody profile and treatment of the 100 patients who underwent vascular US.

4.4.1.2: General vascular US findings – IMT and plaque presence

Plaque presence

36 patients were found to have plaque and a total of 84 plaques were identified. 22 patients had plaque in one or two sites and 15 were found to have plaque in at least three of the four territories assessed. 14% of patients had plaque exclusively in the carotids and in 7% of patients plaque was only found in the femoral bifurcation. 5% of patients had plaque in all four territories assessed.

Patients found to have plaque were significantly older (mean age 53.9; SD 8.76; range 27-66) than those without plaque (mean age 40.0; SD 11.38; range 20-66) ($p < 0.0001$) but no associations with gender or ethnicity were found.

The overall mean plaque score classified using a visual scale was 3.1 (SD 0.91; range 1-5) but the majority of plaques had visual scores between 1 and 3.

Full plaque characterization including area, volume and texture will be explored in the next section.

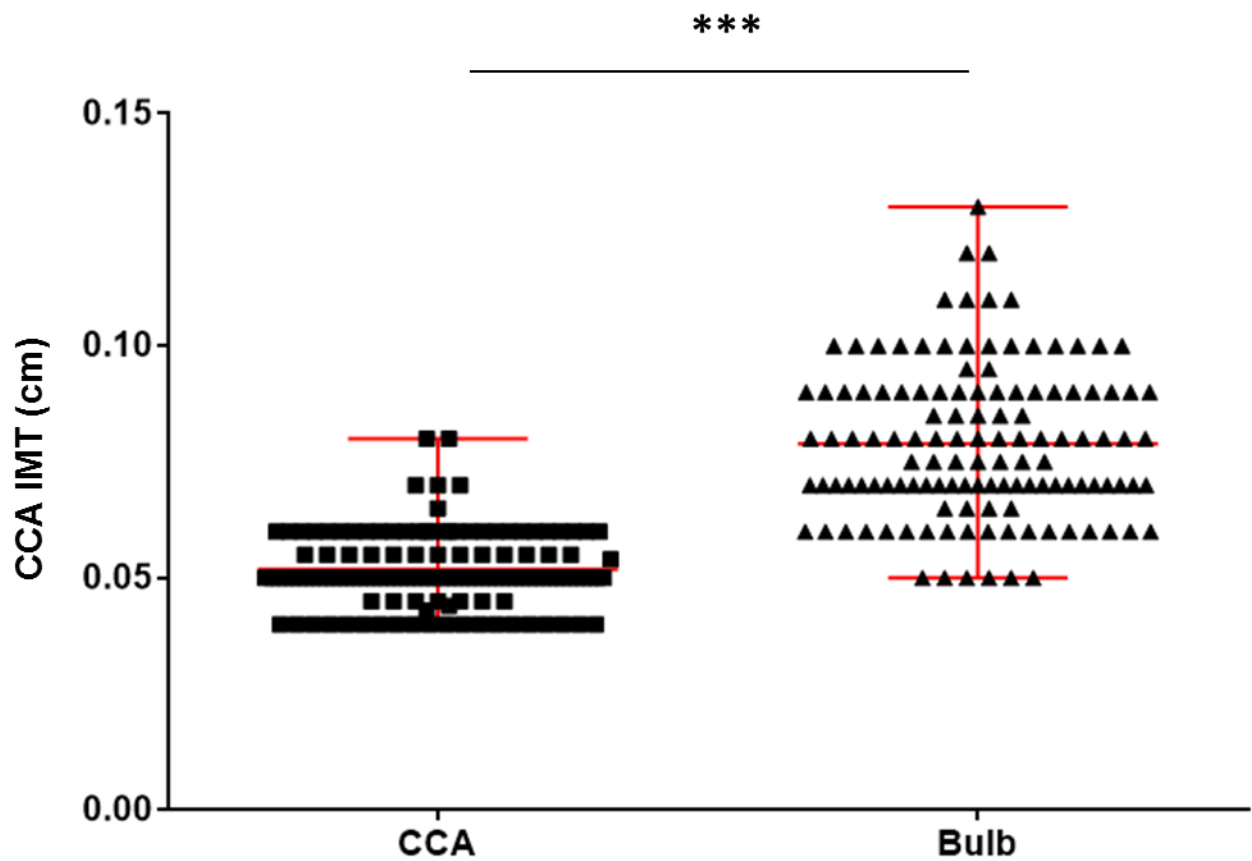
IMT measurements

My findings regarding the IMT measurement taken in the CCA and bulb, as well as the mean CCA IMT and mean overall IMT are summarized in Table 17.

When considering the IMT measured in the CCA, I found that none of the patients scanned had thickened IMT at this level, i.e. all patients had an IMT of less than 0.1cm on both CCAs. Notwithstanding this observation, IMT thickness was significantly greater in the bulb region compared to the CCA even in patients with no plaque anywhere in the carotid territory (Graph 11).

Measurement site	IMT (cm)	SD	Range
Right CCA	0.053	0.009	0.040-0.080
Right Bulb	0.110	0.070	0.050-0.420
Left CCA	0.056	0.050	0.040-0.100
Left Bulb	0.120	0.075	0.050-0.350
Mean CCA IMT	0.054	0.008	0.040-0.088
Mean overall IMT	0.087	0.038	0.045-0.210

Table 17: IMT measurements taken on the mid-section of the CCA and carotid bulb. Mean CCA IMT and mean overall IMT are also included (CCA: common carotid; SD: standard deviation).



Graph 11: IMT in the CCA and carotid bulb: comparison of the IMT thickness between the CCA and bulb region in patients without plaque, showing that the IMT measured at the bulb is significantly greater than at the CCA even in the absence of plaque anywhere in the carotid territory. (***) $p < 0.001$)

Although none of the patients had plaque in the CCA i.e. they all had normal IMT measurements at this level, when I compared the IMT thickness in the CCA between patients without plaque and those with plaque at any site, I found that the latter had significantly thicker CCA IMT ($p < 0.0001$) (Figure 27-A).

I then decided to investigate whether different plaque locations had any influence on this finding and compared the CCA IMT between patients with no plaque and with patients who had plaque exclusively either at the carotid bulb or the femoral bifurcation (Figure 27-B). I found that regardless of the location of plaque, patients with plaque had significantly thicker CCA IMT compared to patients without plaque ($p < 0.0001$ and $p < 0.0033$ respectively).

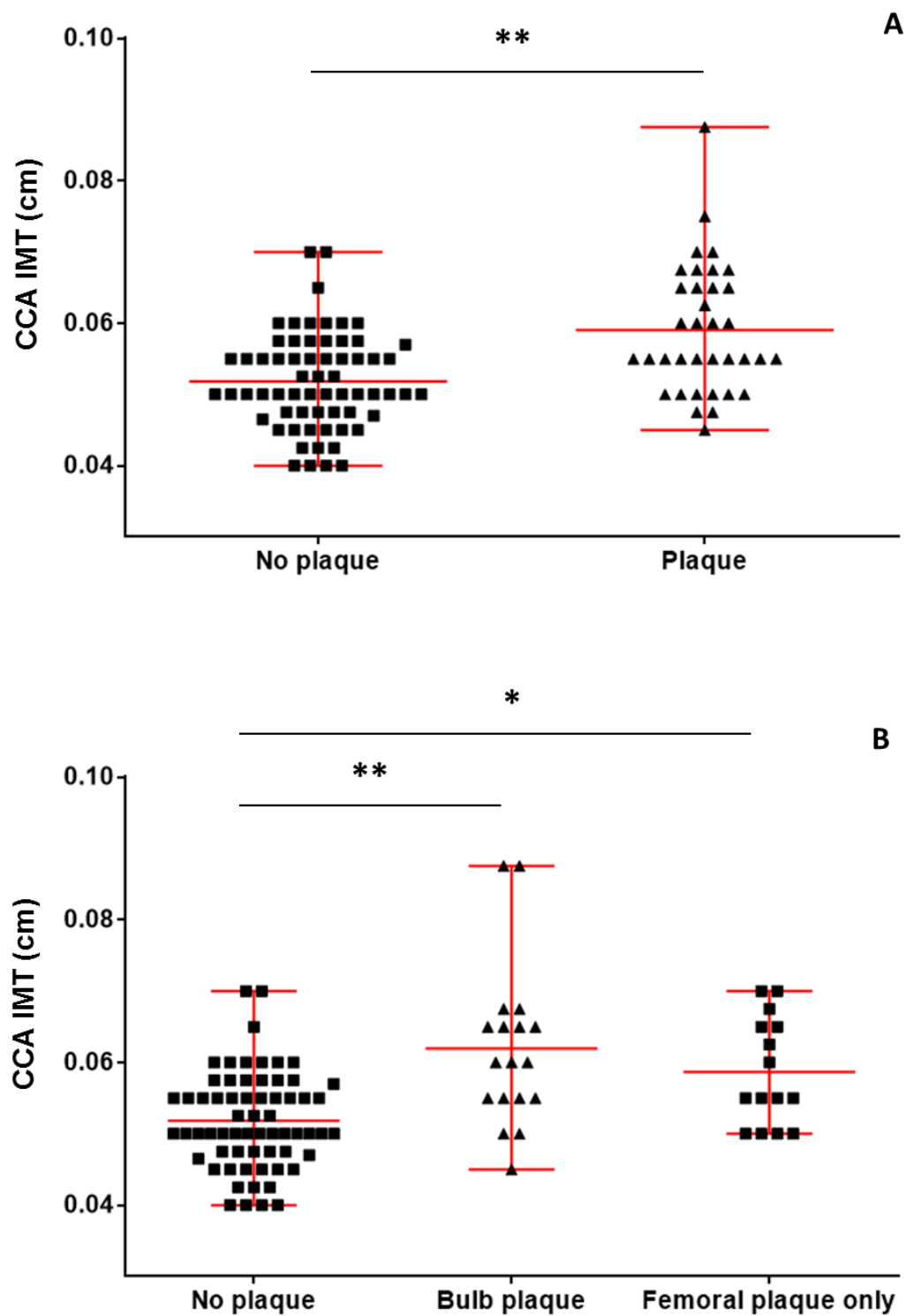


Figure 26: Comparison of the IMT thickness in the CCA between patients without plaque and patients with plaque on any site (A) and plaque exclusively on the bulb or on the femoral bifurcation (B). (* $p < 0.05$; ** $p < 0.01$)

Considering the full patient characterization, several variables appeared to correlate significantly with the mean CCA IMT. Table 18 summarizes these findings.

Both disease duration and age at scan appear to correlate with thicker CCA IMT, in particular the latter. The same is true for BP, with all three measurements having a significant association with the IMT, in particular the systolic BP.

Another interesting observation was the positive correlation found between mean CCA IMT and the number of sites with plaque, total plaque area and total plaque thickness. This reiterates the previously described finding that patients with plaque anywhere had significantly thicker IMT than those without plaque (Graph 19-B).

The lipid profile also had a significant influence over the mean CCA IMT with higher total cholesterol and LDL levels being associated with higher mean CCA IMT. No association between TG and HDL was found, namely, no inverse negative correlation or even trend was found between HDL levels and IMT.

Finally, of the serological tests I developed, the only one for which a weak correlation with mean CCA IMT was found was the anti-ApoA1 IgG level in early disease (but not IgG anti-apoA1 at the time of the sample).

No further statistically significant correlations were found to influence the mean CCA IMT.

Variables	r ² (Spearman correlation; 95% CI)	p- value
Age at scan (yrs)	0.55 (0.7 – 1.4)	<0.0001
Disease duration at scan (yrs)	0.39 (1.2 – 2.0)	0.02
BP (mmHg)		
Systolic	0.4 (1.2 – 2.6)	<0.0001
Diastolic	0.22 (0.8 – 1.7)	0.03
Mean	0.32 (1.1 – 2.4)	0.001
Number sites with plaque	0.39 (0.8 – 1.6)	<0.0001
Total plaque area (mm ²)	0.40 (1.7 – 3.1)	<0.0001
Total plaque thickness (cm)	0.40 (1.4 – 2.7)	<0.0001
T-cholesterol (mmol/L)	0.36 (1.1 – 2.7)	0.0002
LDL (mmol/L)	0.31 (1.4 – 2.3)	0.002
Early anti-ApoA1 IgG (AU)	0.24 (1.0 – 2.1)	0.02

Table 18: Factors significantly influencing the mean CCA IMT (Spearman's correlation; CI 95%)

4.4.1.3: Plaque characterization

4.4.1.3.1: Plaque area and 2D texture analysis

Considering the 36 patients who were found to have plaque on at least one site, the mean total plaque area was 78.67 mm² (SD 69.75; range 6.89-317.93) and the mean total plaque thickness was 0.67 cm (SD 0.07; range 0.16-2.78). All the 84 plaques identified were included in this analysis.

To investigate the variables which had a statistically significant influence on plaque area and thickness, Spearman's correlation was applied. The positive findings of this analysis are summarized in Table 19. Overall both these outcomes were affected in a similar manner by similar variables, although subtle differences were noted.

Variables	Total plaque area (mm ²) (r ² ; 95% CI)	p-value	Total plaque thickness (mm) (r ² ; 95% CI)	p-value
Age at scan (yrs)	0.56 (2.1 – 3.9)	<0.0001	0.58 (1.9 – 3.6)	<0.0001
Disease duration (yrs)	0.29 (1.2 – 2.7)	0.004	0.32 (1.3 – 2.5)	0.0001
Systolic BP (mmHg)	0.27 (1.0 – 2.3)	0.007	0.29 (1.1 – 2.8)	0.004
US findings				
N sites with plaque (n)	0.99 (2.0 – 3.2)	<0.0001	0.99 (2.0 – 3.0)	<0.0001
US class	0.93 (1.9 – 3.0)	<0.0001	0.93 (2.2 – 4.1)	<0.0001
Cumulative AUS	0.84 (1.5 – 4.1)	<0.0001	0.85 (1.2 – 3.7)	<0.0001
Mean CCA IMT (cm)	0.39 (1.7 – 3.2)	<0.0001	0.39 (1.3 – 3.5)	<0.0001
Anti-La positivity	-0.33 (0.7 – 1.3)	0.001	-0.33 (1.0 – 1.9)	0.001
Disease activity (BILAG)				
Persistently moderate/high	0.21 (0.5 – 1.0)	0.035	0.18 (0.8 – 1.1)	0.07
Persistently low	-0.21 (0.6 – 0.9)	0.035	-0.18 (0.4 -0.9)	0.07
N visits with A flare in at least one system (n)	0.23 (1.0 – 1.3)	0.023	0.21 (1.1 – 1.6)	0.03
Homocysteine (μmol/L)	0.31 (0.8 – 1.7)	0.04	0.31 (0.4 – 1.0)	0.05
Triglycerides (mmol/L)	0.27 (1.2 – 2.0)	0.007	0.28 (1.2 – 2.7)	0.005
t-cholesterol:HDL	0.25 (1.1 – 2.0)	0.013	0.23 (1.0 – 1.6)	0.02
Anti-HDL IgG (AU)	0.21 (1.2 – 1.8)	0.03	0.21 (0.7 – 1.2)	0.039
Anti-CCP IgM (AU)	-0.26 (1.0 – 1.9)	0.02	-0.23 (1.0 – 1.6)	0.04

Table 19: Factors significantly associated with total plaque area and thickness (Spearman's correlation; CI 95%).

Similar to what I found for the mean CCA IMT, both age and disease duration at scan were found to correlate with larger and thicker plaques. While neither the dBp nor the mBP appear to correlate with either plaque area or thickness, the sBP was weakly correlated with both variables.

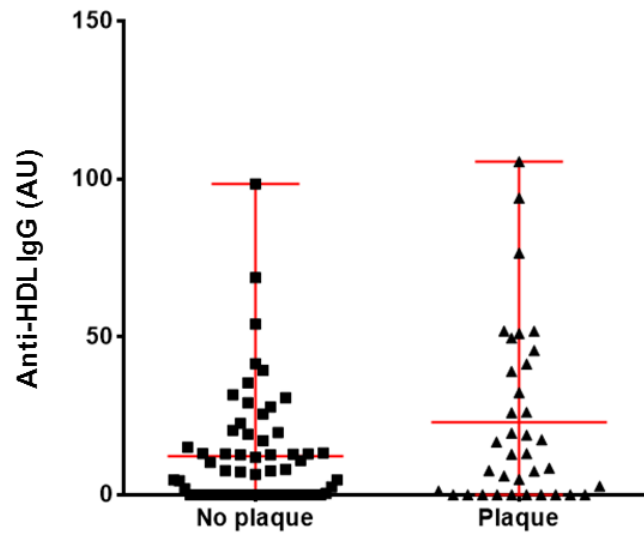
A striking positive correlation between the number of sites with plaque, US class and cumulative AUS and both plaque area and thickness was observed and a similar, although weaker correlation was found with the mean CCA IMT.

When considering the general biochemical laboratory profile, I found that homocysteine had the strongest positive correlation with both plaque area and thickness, although both TG and t-cholesterol:HDL ratio also showed a statistically significant association. With regards to auto-antibody profile, only anti-La positivity emerged as relevant, with a negative correlation found between this and plaque area and thickness. No other ENA subtypes had any significant correlation with these two variables.

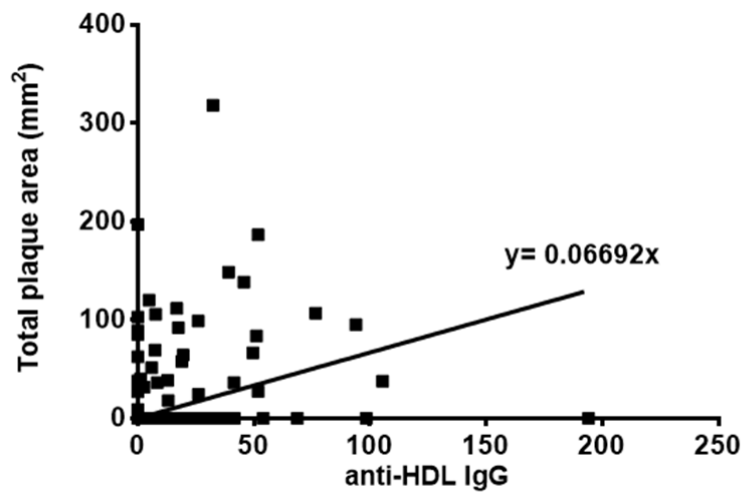
Interestingly, clinically defined disease activity was found to significantly correlate with plaque area but not thickness. Patients who had persistently moderate/ high disease activity tended to have greater plaque area than those with persistently low disease activity. Moreover, the number of visits in which an A flare in at least one of the organ systems considered was recorded was found to have a positive correlation with both plaque area and thickness.

A non-significant positive trend between plaque presence and anti-HDL IgG is shown in Graph 12. Plaque size-related variables (area and thickness) were found to correlate positively with anti-HDL levels (Graph 13).

No statistically significant associations with either anti-ApoA1 IgG or IgM, tApoA1 or nitrated ApoA1 were found (Figure 28).



Graph 12: Comparison of anti-HDL IgG levels between patients with and without plaque.



Graph 13: Plaque area and anti-HDL antibody levels: there is a statistically significant correlation between total plaque area and anti-HDL IgG levels ($p=0.033$). The line represents the linear regression and its equation (r^2 0.56).

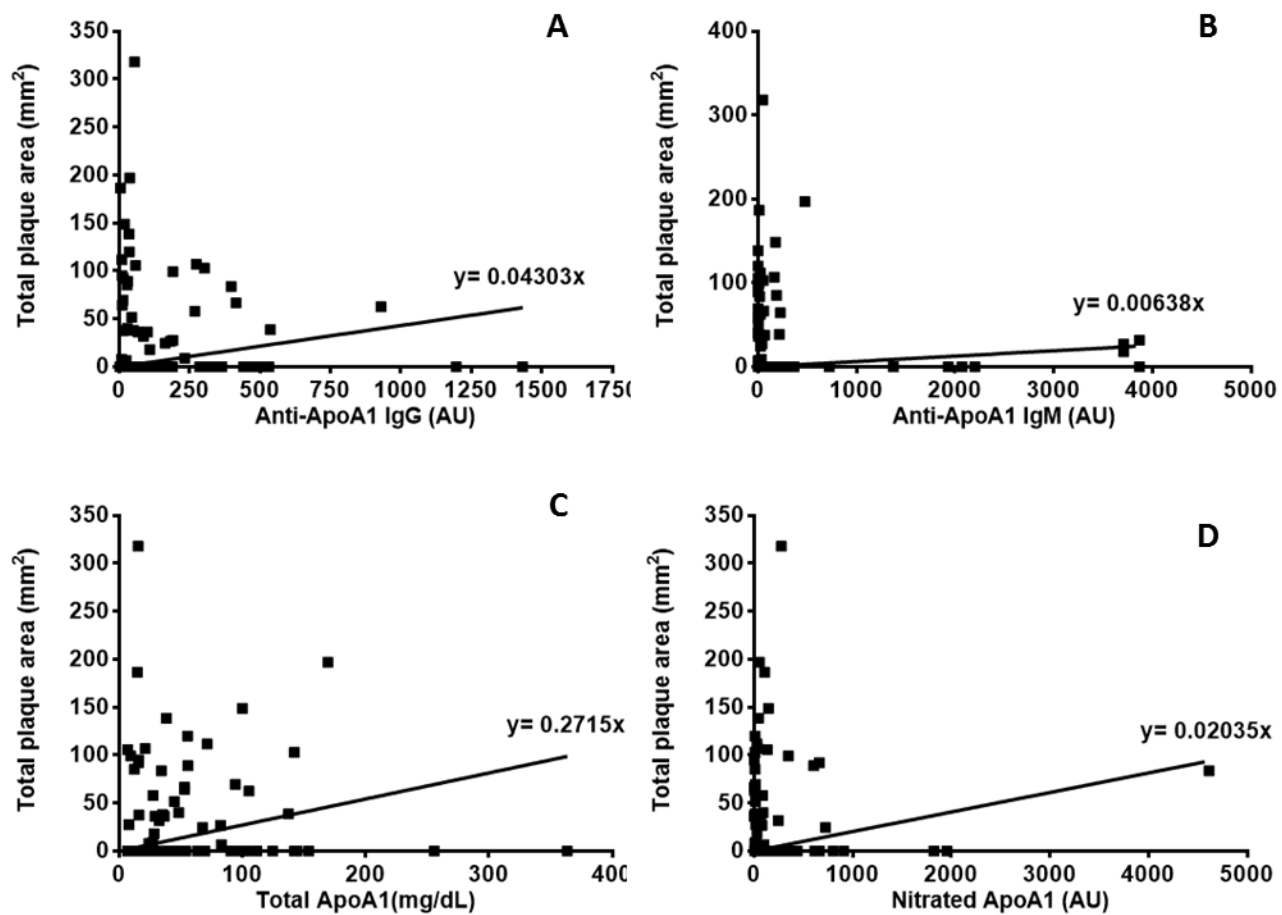


Figure 28: Correlation between plaque area and anti-ApoA1 IgG (A) and IgM (B), total ApoA1 (C) and nitrated ApoA1 (D). The line represents the linear regression and its equation. Statistical significance was not found for any of these variables (A r^2 0.1; B r^2 0.16; C r^2 0.32; D r^2 0.09).

Finally, with regards to plaque texture analysis, I found that based on the qualitative scale (0-5), the average value was 3, which represent plaques that are mainly echolucent although there is also a significant connective tissue component. The majority of the plaques were heterogeneous and discrete white areas were identified in two thirds. The predominance of echolucent but heterogeneous plaques was further emphasized when a quantitative scale, the GSM was applied. The GSM of the plaques identified in the vascular US cohort did not follow a normal distribution as shown on Figure 29. The median GSM value was 48.9 (IQR 34.3-59.0). Considering that the GSM represents a spectrum of echogenicity where the lower the values (from 0) represent maximum echolucence, i.e. greater lipid content, while the higher values describe greater echogenicity (high calcium/ fibrotic tissue content), my findings support the concept that the majority of plaques are predominantly echolucent as suggested by the qualitative visual scale previously described.

The factors found to influence GSM significantly are summarized on Table 20.

A positive association between renal function and GSM was found with serum creatinine and to a lesser extent serum urea found to significantly correlate with GSM. No correlation between GSM and urine protein: creatinine ratio was found.

Sustained disease activity was also found to influence GSM. Patients who had persistently moderate/ high disease activity were found to have higher GSM values. An inverse correlation was observed for patients who had persistently low disease activity.

Although serological markers of disease activity (anti-dsDNA and C3) were not found to influence GSM, the presence of anti-CCP antibodies appeared to correlate negatively with GSM while the presence of anti-ApoA1 IgM had a positive correlation with this outcome. However, when looking at the total serum IgM level, this was also found to correlate with GSM in a similar manner and therefore, this could be a potentially confounding factor. The total serum IgA levels were found to correlate inversely with GSM.

Finally, a non-statistically significant positive trend between total plaque area and GSM was found ($p= 0.06$) but not with total plaque thickness ($p= 0.388$).

Neither total ApoA1, nitrated ApoA1, age at scan, disease duration or lipid profile were found to significantly influence GSM.

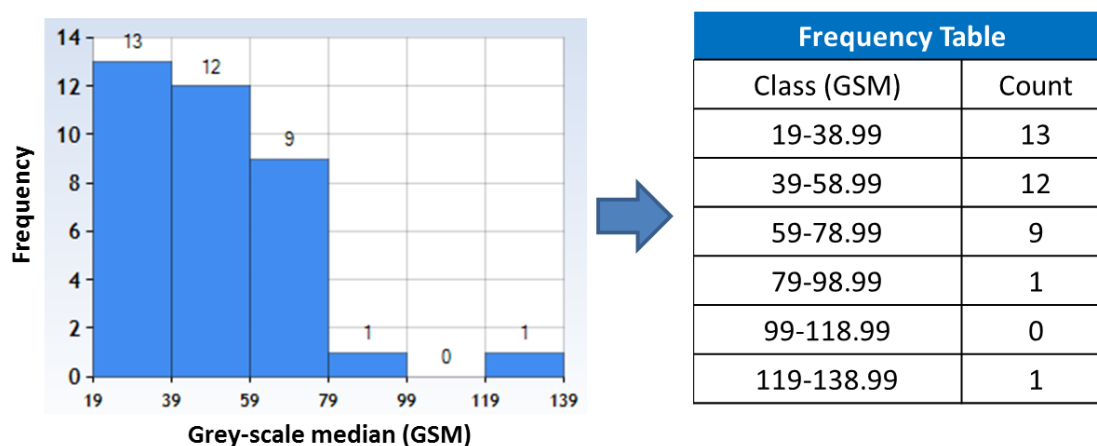


Figure 29: Histogram and table of frequencies showing the GSM distribution in the vascular US cohort.

Variables	GSM (r^2 ; 95% CI)	p-value
Sustained disease activity		
moderate/high	0.52 (1.3 – 2.1)	0.001
low	-0.52 (1.5 – 2.3)	0.001
Renal function		
Urea (mmol/L)	0.33 (0.9 – 1.4)	0.05
Creatinine (μmol/L)	0.46 ((1.1 – 2.0)	0.005
Anti-C1q positivity	0.44 (1.2 – 2.9)	0.02
Serum IgM (mg/dL)	0.35 (1.0 – 2.7)	0.04
Serum IgA (mg/dL)	-0.39 (0.8 – 1.6)	0.02
Anti-apoA1 IgM (AU)	0.35 (0.7 – 1.1)	0.04
Anti-CCP positivity	-0.46 (1.6 – 3.0)	<0.0001
Anti-ApoA1 IgM (AU)	0.35 (1.1 – 1.9)	0.04

Table 20: Factors found to be significantly correlated with GSM (Spearman's correlation; CI 95%).

4.4.1.3.2: Plaque volume and 3D texture analysis

As previously established, at present, only carotid plaques can be adequately assessed by 3D US. Therefore, of the 84 plaques identified in 36 patients, only 40 plaques were included in this section. An additional two carotid plaques were identified, but due to heavy calcification or anatomical constraints, image analysis was not feasible and they were excluded.

The mean volume of the carotid plaques considered was 37.79mm^3 (SD 35.7; range 2.52-119.64). Table 21 shows the variables found to significantly influence plaque volume on univariable analysis.

Similar to what was previously described regarding plaque area, both age and disease duration on the scan were found to correlate with plaque volume. The same applies to the influence of BP, with sBP as the only significant correlation.

As previously noted for total plaque area and thickness, the general vascular US findings (number of sites with plaque, US class and cumulative AUS score) were shown to influence plaque volume significantly and a strong correlation between plaque area, thickness and volume was also found.

With regards to serological findings, anti-HDL IgG was found to have a positive correlation with plaque volume while both anti-La and anti-ApoA1 IgM positivity were noted to have a negative correlation.

No other statistically significant associations were found, with renal function, lipid profile or disease activity.

Variables	Total plaque volume (mm ³) (r ² ; 95% CI)	p-value
Age at scan (yrs)	0.56 (1.3 – 2.4)	<0.0001
Disease duration (yrs)	0.23 (1.0 – 1.8)	0.02
Systolic BP (mmHg)	0.23 (1.3 – 2.1)	0.02
US findings		
N sites with plaque	0.83 (2.4 – 3.6)	<0.0001
US class	0.80 (2.5 – 4.0)	<0.0001
Cumulative AUS	0.71 (2.5 – 3.2)	<0.0001
Mean CCA IMT (cm)	0.44 (1.7 – 3.1)	<0.0001
Total plaque area (mm ²)	0.85 (0.6 – 1.9)	<0.0001
Total plaque thickness (cm)	0.84 (1.2 – 2.1)	<0.0001
Anti-La positivity	-0.27 (1.2 – 2.1)	0.006
Anti-dsDNA (IU/L)	-0.21 (1.0 – 1.9)	0.04
Anti-CCP IgM (AU)	-0.30 (1.3 – 2.5)	0.004
Anti-HDL IgG (AU)	0.24 (1.5 – 2.1)	0.02

Table 21: Factors found to significantly correlate with total plaque volume (Spearman's correlation; CI 95%).

Considering plaque texture analysis using volume assessment (GSMvol), I found very few variables whose influence was statistically significant and this could, at least partly be due to the relatively small number of cases assessed. A summary of these findings is shown in Table 22.

A significant positive correlation was found between volume-based GSM (GSMvol) and 2D US-based GSM as Graph 14 depicts. Similar to what was found for GSM, disease activity also appeared to significantly influence GSMvol although the strength of this correlation was weaker than that previously noted.

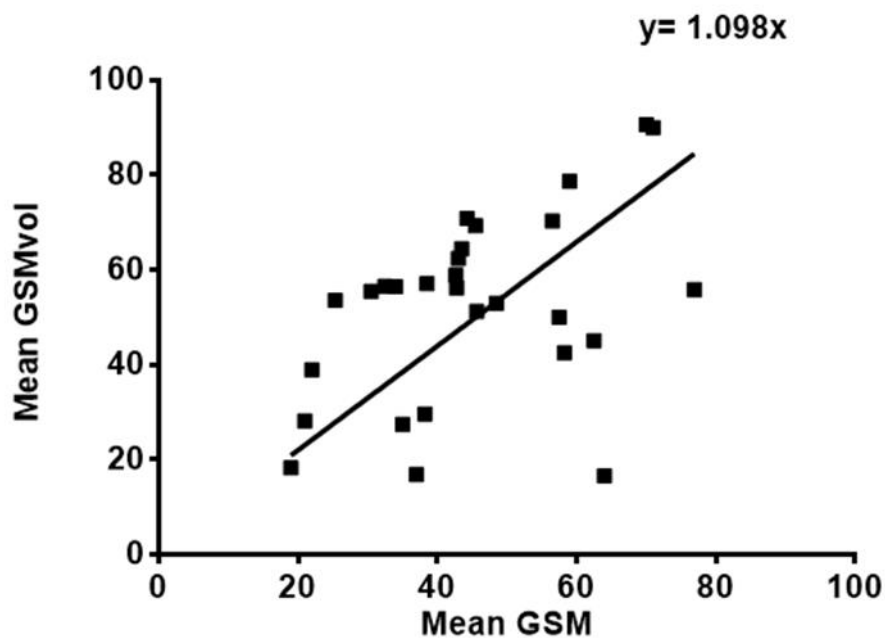
Positivity for anti- β 2GP1 antibodies and lupus anticoagulant was found to have a significant negative correlation with GSMvol. As described for GSM, an inverse correlation with anti-CCP positivity was also found.

A non-significant negative trend between the levels of HDL and GSMvol was found but the levels of anti-HDL or anti-ApoA1 antibodies did not influence this outcome.

No other serological variables were found to influence GSMvol significantly, such as renal function, serum immunoglobulin levels, lipid or remaining auto-antibody profile.

Variables	GSMvol (r^2 ; 95% CI)	p-value
Mean GSM	0.38 (1.2 – 2.5)	0.04
Sustained disease activity		
moderate/high	0.38 (1.1 – 1.9)	0.049
low	-0.38 (0.9 – 1.4)	0.049
Anti- β 2GP1 positivity	-0.44 (1.2 – 2.0)	<0.0001
Lupus anticoagulant positivity	-0.39 (1.0 – 2.1)	0.02
Anti-CCP positivity	-0.30 (0.8 – 1.7)	<0.0001

Table 22: Factors found to correlate significantly with GSMvol (Spearman's correlation; CI 95%).



Graph 14: Correlation between 2D US-based GSM (GSM) and volume-based GSM (GSMvol). The line depicts the linear regression and its equation ($n=40$, r^2 0.69, $p=0.02$).

4.4.1.4: Comparison of vascular US findings between a SLE and a non-SLE cohort

In order to compare the vascular US findings from the cohort of patients with SLE with a non-SLE cohort, the patients were age and sex matched with non-SLE patients selected from the Cyprus epidemiological study as previously described. For each of the patients with SLE, two matched controls were found ($n= 132$). Due to the fact that the Cyprus cohort excluded individuals aged below 40-years of age, adequate matching was only possible for 66 of my patients with SLE. The data presented in this section will therefore only consider this sub-section of the SLE cohort.

Figure 30 depicts the age histograms for both populations showing a normal distribution while Table 23 summarizes the age and sex distribution as well as the general vascular US findings for the two groups.

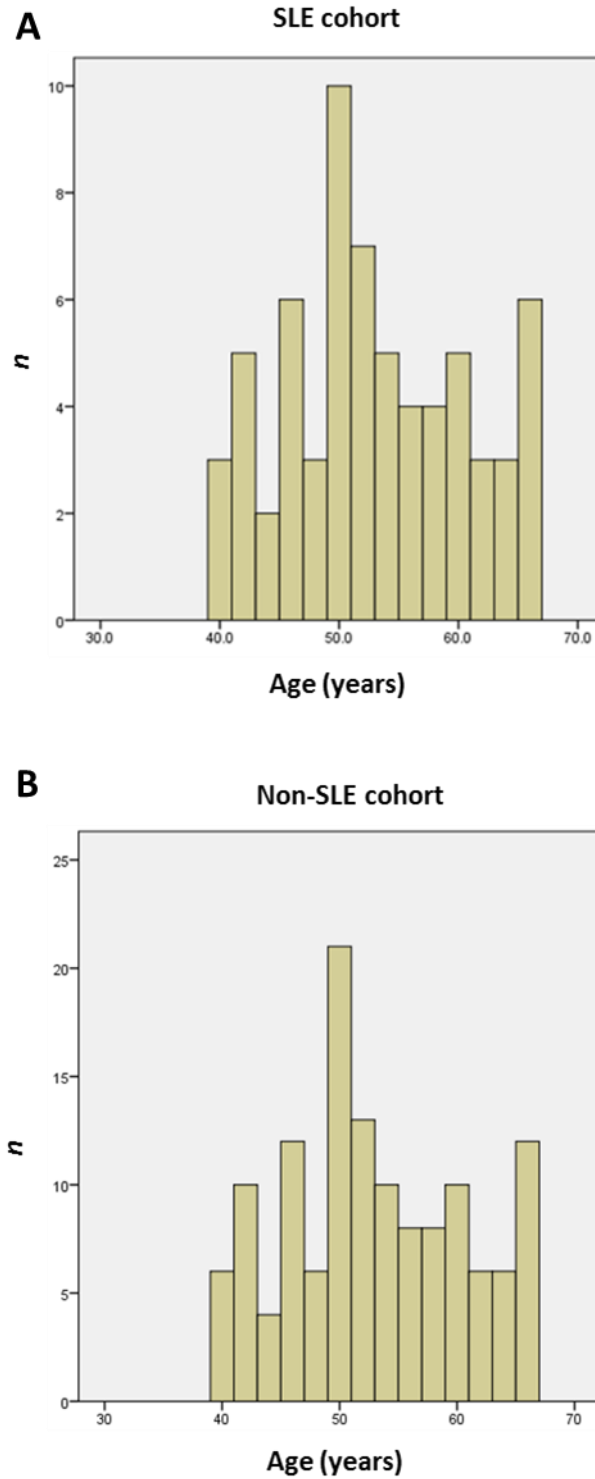


Figure 30: Histograms representing the age distribution for the SLE cohort (A) and the age and sex matched non-SLE controls selected from the Cyprus epidemiological study (B).

Variables	SLE cohort – mean values (n= 66)	SD (range)	Non-SLE cohort – mean values (n= 132)	SD (range)
Sex (n Female : Male)	63 : 3	-	126 : 6	-
Age at scan (years)	52.6	7.6 (40-66)	52.6	7.5 (40-66)
N sites w/ plaque	1.2	1.4 (0-4)	0.82	1.2 (0-4)
Cumulative AUS	11.3	7.8 (0-26)	9.1	6.6 (0-28)
Mean CCA IMT (cm)	0.057	0.008 (0.04-0.09)	0.064	0.01 (0.035-0.095)
Total plaque thickness (cm)	0.30	0.37 (0-1.41)	0.17	0.26 (0-1.14)
Total plaque area (mm²)	41.8	60.9 (0-317.9)	14.34	25.9 (0-128)
Mean plaque GSM	48.6	21.6 (19-128)	14.6	10.8 (0.25-40.25)

Table 23: Comparison between sex and age distribution and general vascular US findings between the SLE and non-SLE cohorts.

Age and sex matching between the two groups was successfully achieved. Although the cumulative AUS score was not significantly different in the two cohorts, the number of sites with plaque was significantly greater in patients with SLE compared with the non-SLE cohort ($p= 0.0047$).

As shown in Figure 31-A, the mean CCA IMT was significantly greater in the non-SLE group. However, total plaque thickness and total plaque area were markedly increased in the SLE patients compared with the non-SLE group (Figure 31-B and C respectively).

Significant differences were also noted regarding plaque texture: GSM was significantly greater in the SLE group compared with non-SLE controls as shown on Graph 15.

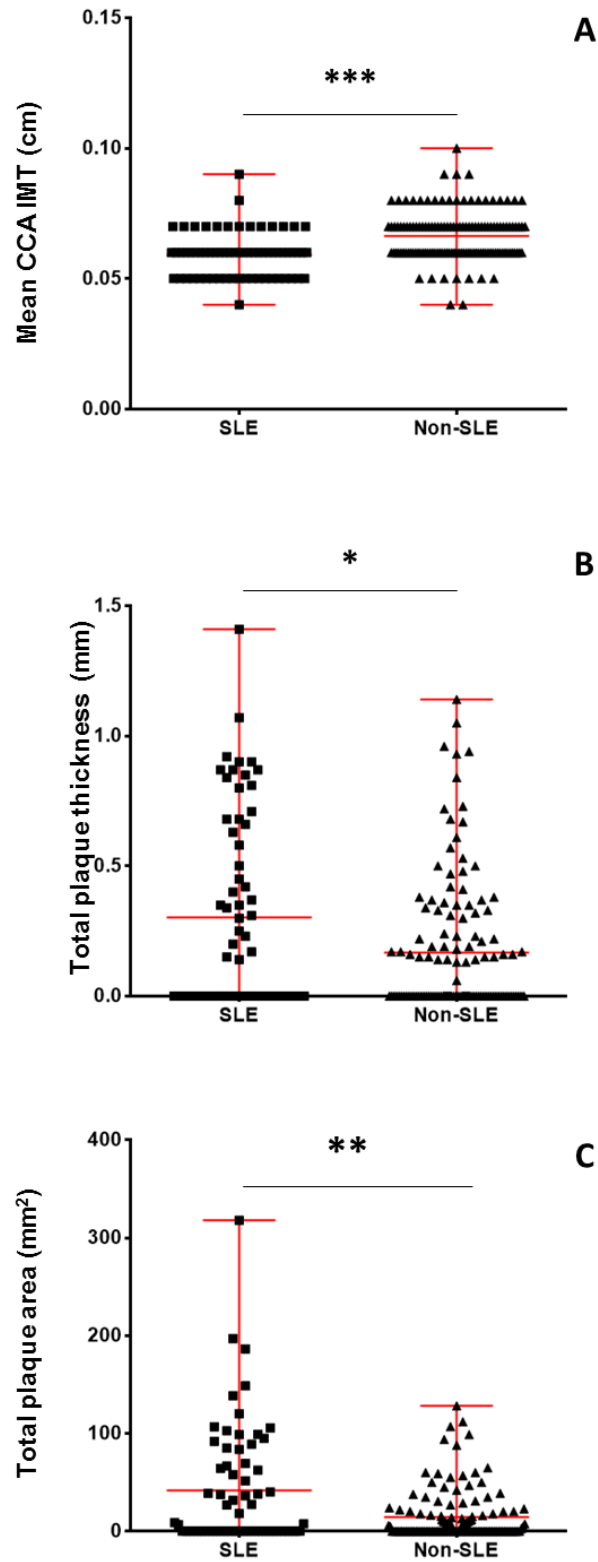
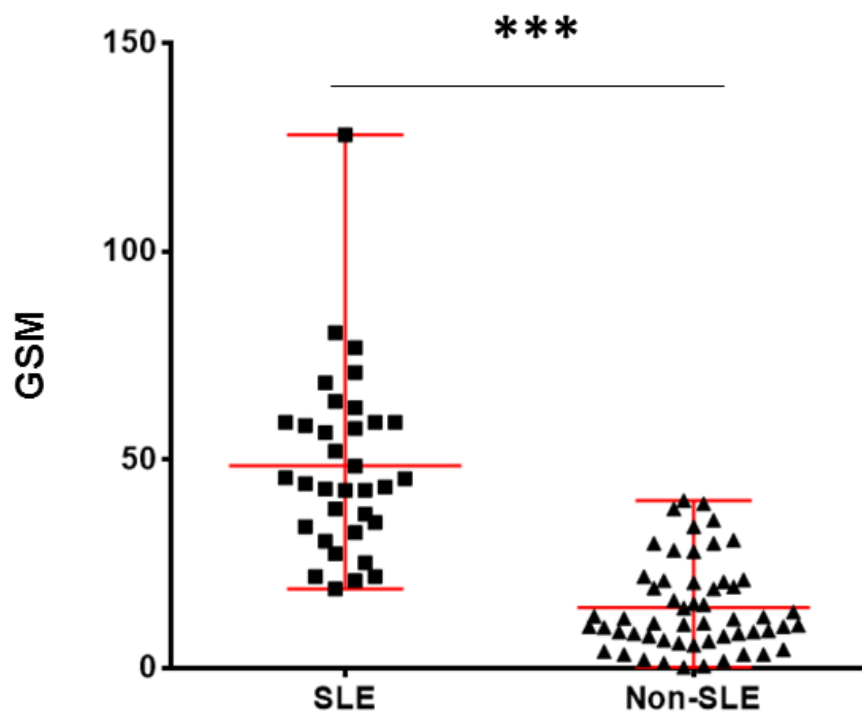


Figure 31: Comparison between the mean CCA IMT (A), total plaque thickness (B) and total plaque area (C) between the SLE and non-SLE cohorts.
 (* $p < 0.05$; *** $P < 0.001$)



Graph 15: Comparison between the mean GSM between the SLE and non-SLE cohorts.

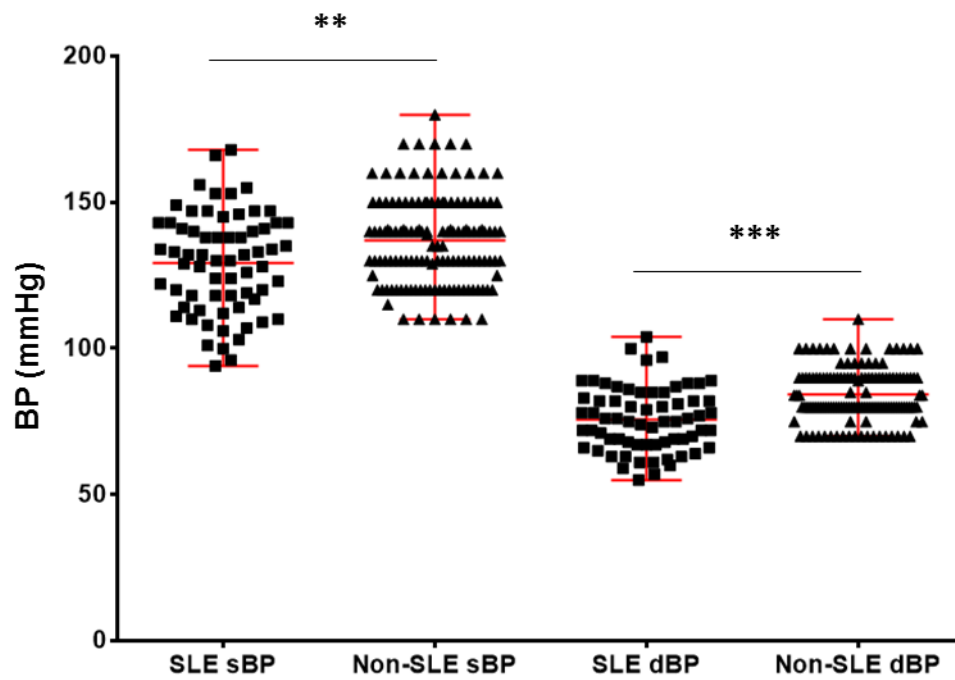
(** $p < 0.01$; *** $P < 0.001$)

When comparing individual characteristics between the two groups, the absolute number of individuals who were smokers at the time of the scan was similar in both cohorts (SLE group n= 11; Non-SLE group n= 13) and a similar proportion of individuals with diabetes was also observed (SLE group n= 2; Non-SLE group n= 4). However, a different picture was observed for blood pressure measurements as those belonging to the non-SLE group had significantly higher sBP and dBP than those noted for patients with SLE ($p= 0.0015$; $p<0.0001$ respectively) as shown on Graph 16.

The lipid profile was also found to be markedly different between the two groups: patients with SLE had significantly lower total cholesterol, triglycerides and LDL levels, but higher HDL levels compared with the non-SLE individuals as show in Figure 32-A, B, C and D respectively.

Although it was still within the normal range for the majority of patients, serum creatinine was significantly higher in the SLE group (Figure 32-E).

Finally, serum homocysteine levels were also markedly higher in patients with SLE compared with the non-SLE individuals (Figure 32-F).



Graph 16: Comparison between the systolic and diastolic blood pressure between the SLE and non-SLE cohorts.

(** $p < 0.01$; *** $P < 0.001$)

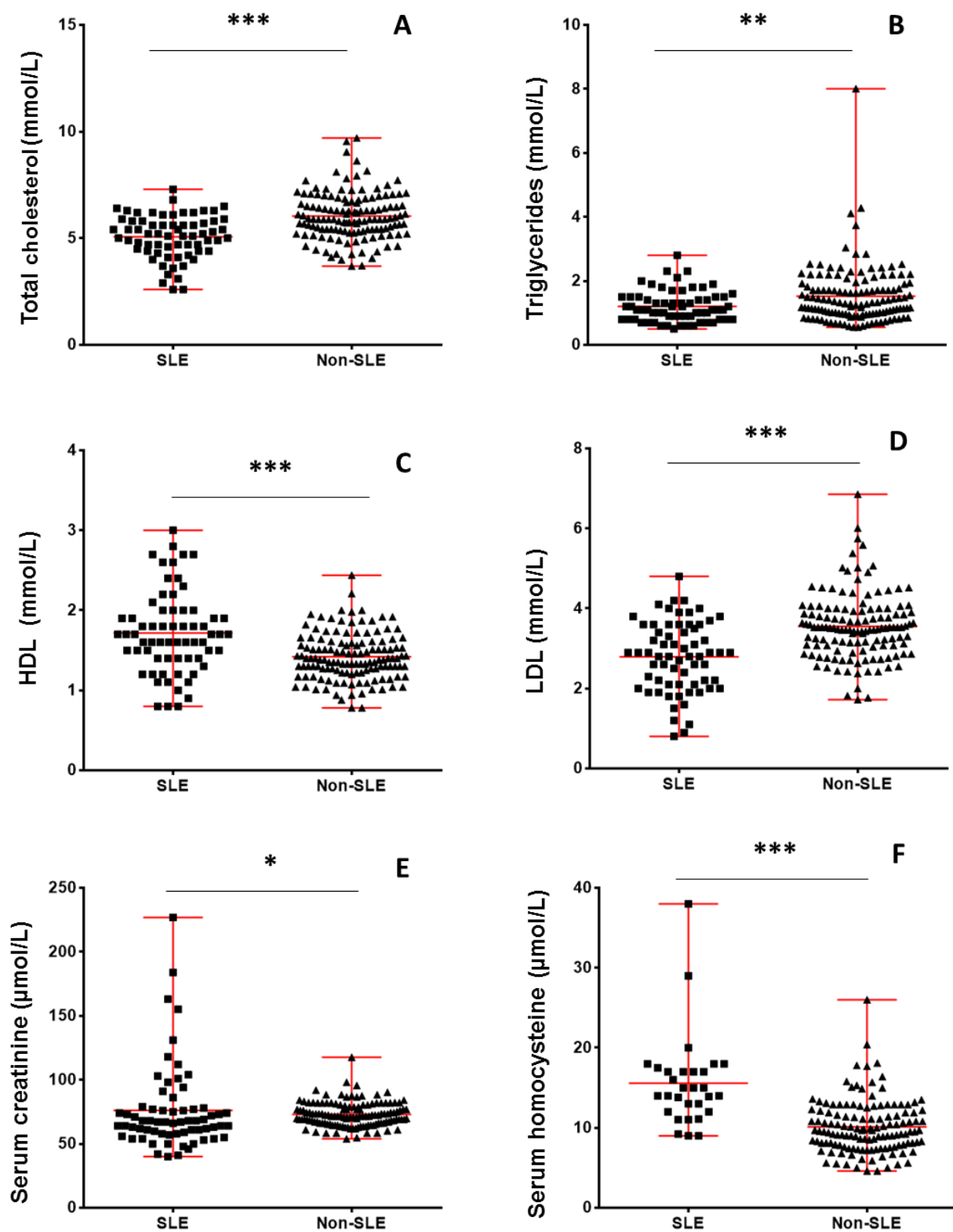


Figure 32: Comparison between SLE and non-SLE groups regarding the total cholesterol (A), triglycerides (B), HDL (C), LDL (D), serum creatinine (E) and serum homocysteine (F).

(* $p < 0.05$; ** $p < 0.01$; *** $P < 0.001$)

Taking into account the hypothesis that patients with SLE may have accelerated atherosclerosis, I explored the correlation between age at scan and both IMT thickness and plaque area using a linear regression model.

As shown on Figure 33-A, no differences were found when plotting age at scan and mean CCA IMT for both groups. However, if total plaque thickness was considered, patients with SLE appear to have a steeper regression line which suggests that the progression of plaque thickness could be happening faster in patients with SLE (Figure 33-B). This is further emphasised if age is plotted against total plaque area as shown on Figure 34, suggesting that there is potentially a different plaque growth pattern in patients with SLE compared with non-SLE controls.

Looking at this hypothesis on a different angle, I compared the correlation between mean CCA IMT and either total plaque thickness or total plaque area and found a similar pattern supporting the notion that plaque progression may be occurring differently in the two groups (Figure 35).

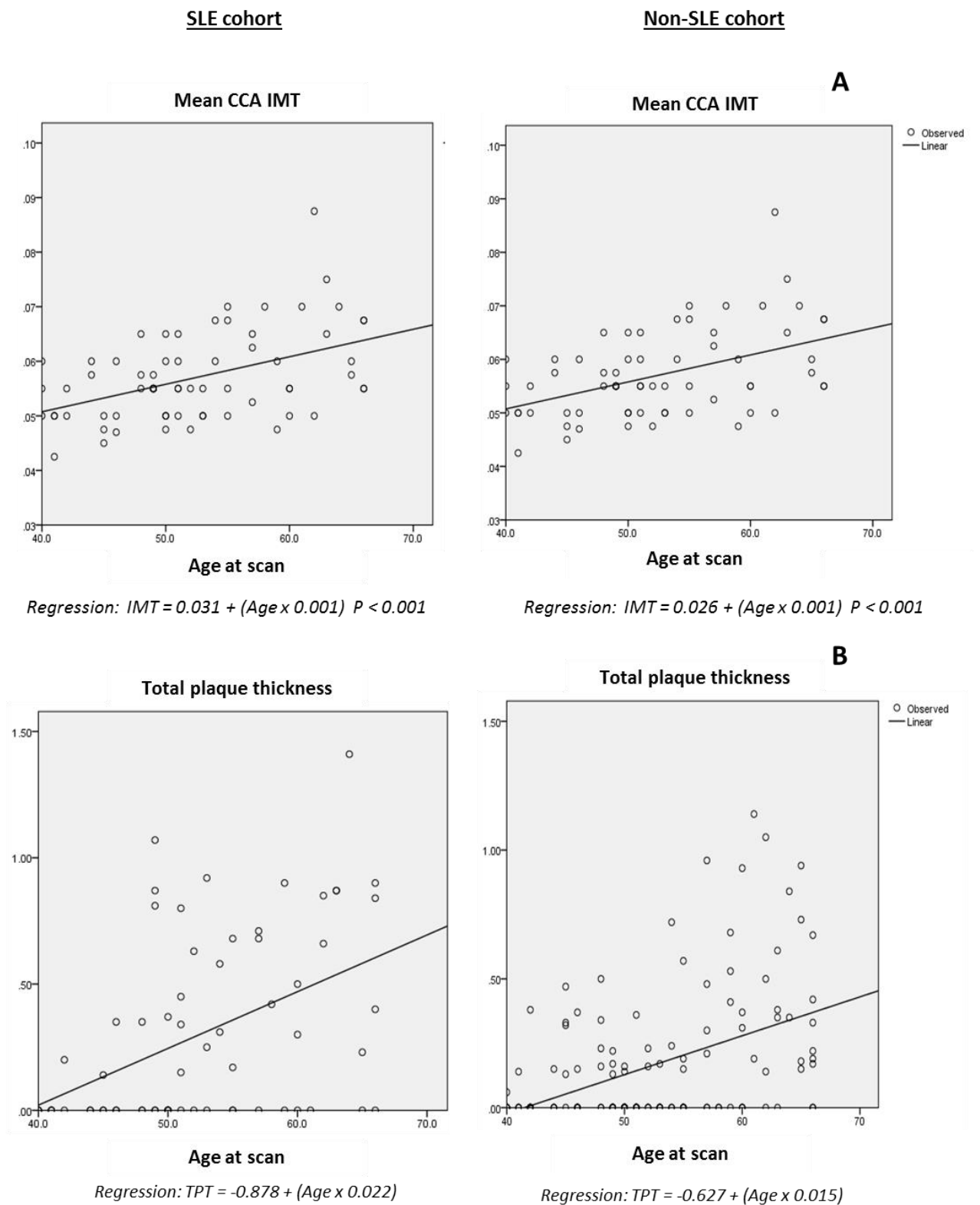


Figure 33: Comparison between SLE and non-SLE groups using a linear regression model depicting age at scan plotted against mean CCA IMT (A) and total plaque thickness (B).

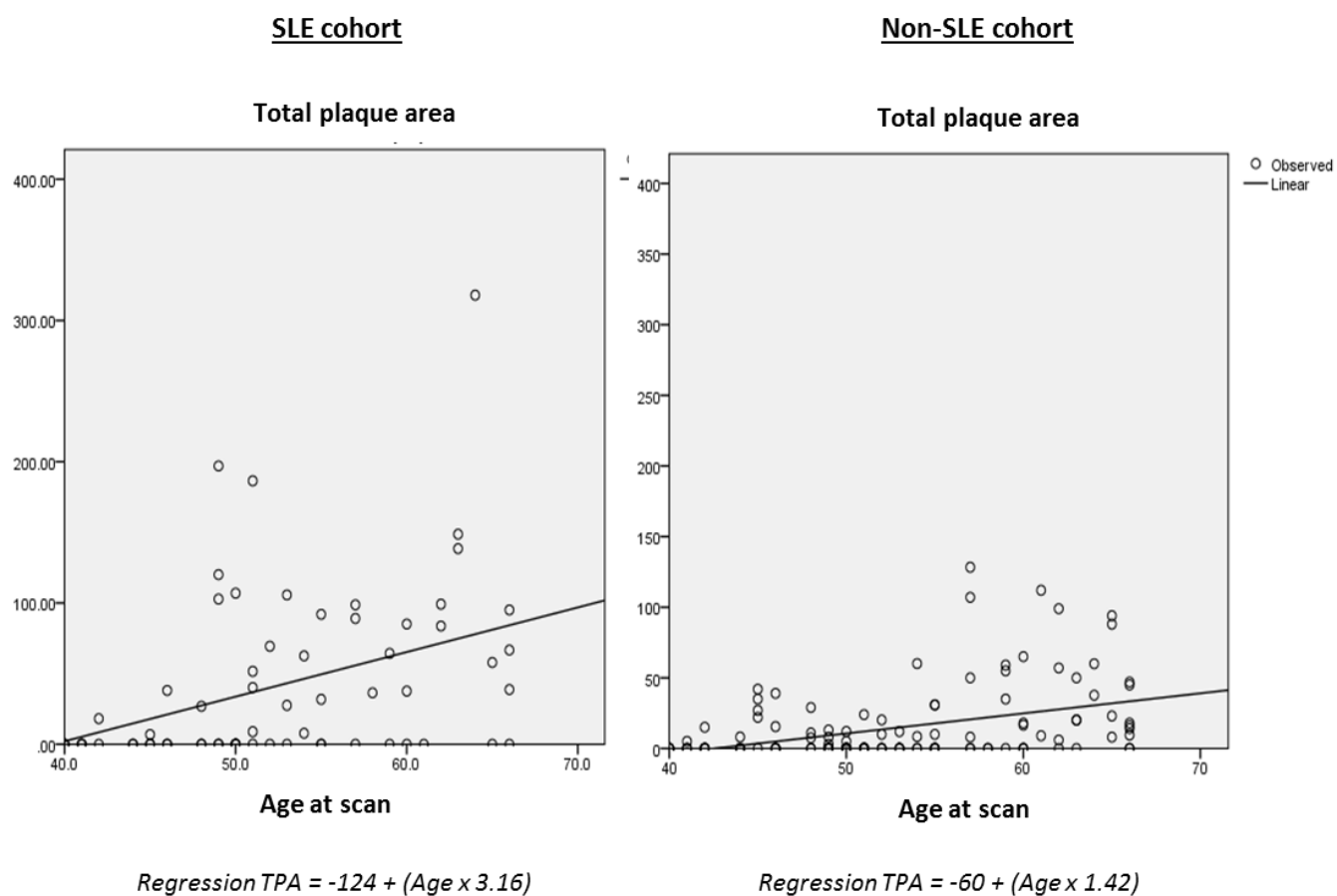


Figure 34: Comparison between SLE and non-SLE groups using a linear regression model depicting age at scan plotted against total plaque area.

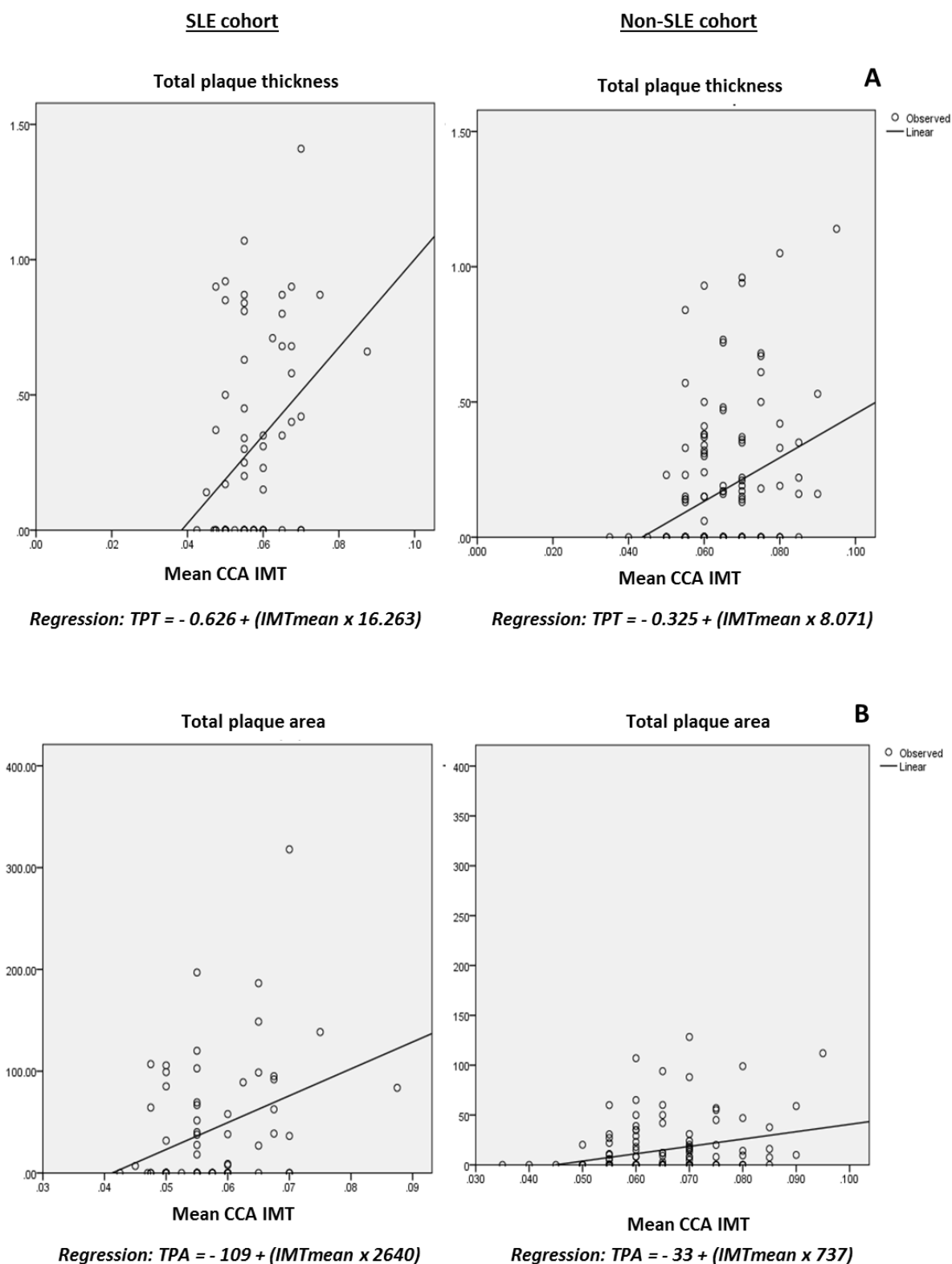


Figure 35: Comparison between SLE and non-SLE groups using a linear regression model depicting mean CCA IMT plotted against total plaque thickness (A) and total plaque area (B).

4.4.1.5: Discussion

SLE has been established as one of the strongest, independent risk factors for CVD (D'Cruz, Khamashta et al. 2007, Pennell and Keenan 2011). However, atherosclerosis is a gradual process and formation of plaque starts well before there is any clinical evidence, i.e. there is a period of subclinical atherosclerotic or CVD (Stary, Chandler et al. 1995). As the majority of patients with SLE score low in the traditional risk scores (Bessant, Hingorani et al. 2004, O'Neill, Pego-Reigosa et al. 2009), they usually don't receive any targeted treatment such as statins until they either develop symptoms suggestive of CVD (namely angina, TIA or intermittent claudication) or have a major CVD-related event (stroke, MI). Therefore, any strategies that are put in place will be, at best, to achieve secondary prevention.

In the SLE cohort, 36 out of 100 patients with SLE with no evidence of CVD already had plaque. The 36% prevalence is in keeping to what has been previously reported by other groups (Manzi, Selzer et al. 1999, Svenungsson, Jensen-Urstad et al. 2001, Doria, Shoenfeld et al. 2003, Roman, Shanker et al. 2003, Selzer, Sutton-Tyrrell et al. 2004, Wolak, Todosoui et al. 2004) and is significantly higher than what has been reported in healthy individuals (Svenungsson, Jensen-Urstad et al. 2001, Roman, Shanker et al. 2003, Thompson, Sutton-Tyrrell et al. 2008).

The fact that I have included the femorals in the US assessment has increased the sensitivity of the method as in 7% of patients, plaque was found exclusively on this location. Although usually in research, only carotids are assessed, given that atherosclerosis is a generalised process, including the femorals increases the chances of identifying plaque. Moreover, the involvement of multiple territories increases the likelihood of coronary involvement. This is supported by my finding that patients who had femoral plaques only, the IMT thickness in the CCA was significantly different from patients with no femoral plaques (Graph 13). This result supports the notion that atherosclerosis involves the arterial bed globally and that the presence of plaque, regardless of its location, signals the generalised presence of on-going endothelial dysfunction/ damage. This is further supported by the fact that patients who had plaque in the bulb region, despite not having IMT greater than 0.1cm, had significantly thicker IMT than those without plaque.

Carotid atherosclerosis is usually the most widely used variable when studying CVD and its correlation with risk of event or different biomarkers. However, there are different studies focusing on peripheral atherosclerosis, namely on the femoral territory which highlight the relevance of considering this vascular bed when establishing a risk profile. Brevetti et al showed how patients with peripheral artery disease had greater atherosclerotic burden and more echolucent plaques compared with those who only had coronary artery disease and

suggested that the presence of femoral plaques, particularly those with reduced GSM may have increased CVD risk, namely of cerebrovascular disease (Brevetti, Sirico et al. 2008). These findings were supported by the work of Schiano et al who assessed the risk of MI and stroke in patients with femoral atherosclerosis and found that the presence of echolucent femoral plaques is associated with increased prevalence of CVD-related events (Schiano, Sirico et al. 2012). The median GSM of the femoral plaques identified in the vascular US cohort was 52. This is relatively low value, which is in keeping with the increased CVD risk found among patients with SLE, further emphasizing the need for a comprehensive vascular assessment in order to optimize risk stratification.

The significant differences found between the IMT thicknesses in the bulb vs. CCA, even in the absence of plaque in either of these sites, have been previously reported and are thought to be due to the normal physiological difference in blood flow between the two sites (Roman, Saba et al. 1992). While in the CCA, the blood flow is laminar, minimizing shear stress forces, in the bulb the blood flow becomes turbulent, therefore stimulating the proliferation of smooth muscle cells.

As summarized in Table 3 of the Introduction, the majority of studies focused on assessing the impact of plaque in defining the risk for CVD-events have shown that the presence of plaque, particularly those with greater area and eliciting a significant luminal stenosis (>50%) is associated with increased risk of ACS, regardless of the CCA IMT value (Salonen and Salonen 1991, Held, Hjerdahl et al. 2001, Hunt, Evans et al. 2001, van der Meer, Bots et al. 2004, Johnsen, Mathiesen et al. 2007). The patients included in the vascular US cohort who were found to have plaque seldom had a luminal stenosis greater than 50% (3/84), but although the plaques did not encroach into the lumen, the total plaque area was significant, suggesting that the plaque growth may occur predominantly along the arterial wall as will be discussed further down.

As previously described, besides the size (area and/or volume), the composition of the plaque has substantial influence in the risk of CVD-related events. The fact that the mean overall qualitative plaque score in the cohort I assessed is 3, shows that in these patients, the plaques found are predominantly cholesterol rich and could potentially be associated with a greater risk of rupture. Despite being useful, the characterization of plaque using a qualitative scale is inevitably subjective and has limited power in distinguishing plaques which have similar echographic appearance. Thus, the use of a quantitative measurement such as the GSM is not only more accurate but also more sensitive in portraying subtle differences in plaque texture (El-Barghouty, Levine et al. 1996, Aly and Bishop 2000, Prahl, Holdfeldt et al. 2010). The GSM ranges from 0 to 250 and the score has a positive

correlation with plaque echogenicity (i.e. echogenic plaques have a high GSM, echolucent plaques have low GSM). The majority of the plaques identified in this SLE cohort have a low GSM, which means that patients have predominately echolucent or lipid rich plaques (Goncalves, Lindholm et al. 2004, Sztajzel, Momjian et al. 2005).

The division of plaques into echolucent versus echogenic as a synonym for collagen-rich versus lipid rich has been increasingly disputed, as new concepts on plaque echogenicity and its correlation with the nature of the echogenic tissue it contains, emerge. Moving from the original view that the presence of echogenic material within the plaque was due either to the presence of calcium and/ or fibrous tissue, Goncalves et al established that the echogenicity of carotid plaque was mainly determined by their elastin and calcium content and only to a lesser extent by collagen or lipids (Goncalves, Lindholm et al. 2004). Other groups have focused on the issue of determining why plaques appear to be echolucent rather than echogenic in US assessment and found that it may also be due to the presence of neovascularization. Several studies of contrast US using microbubbles have shown that the presence of increased intra-plaque vascularization is associated with decreased echogenicity and low GSM, suggesting that the presence of neovascularization may be a relevant feature when determining plaque texture. It is argued that the presence of increased intra-plaque vascularization may lead to increased plaque haemorrhage and consequently increased risk of plaque rupture and thrombosis (Coli, Magnoni et al. 2008, Owen, Shalhoub et al. 2010, Staub, Partovi et al. 2011, Zhou, Xing et al. 2013). In non-contrast US, these micro-blood vessels appear as intensely white, non-calcified regions within the plaque described as “discrete white areas” and when present may correlate with neovascularization (Shah, Falk et al. 1995, Griffin, Kyriacou et al. 2010).

Another factor to take into account when assessing plaque echogenicity is whether its texture is homogenous or heterogeneous. Heterogeneous plaques typically present with echolucent and echogenic areas and are best described as a type 3 plaque; using the qualitative scale this score defines a plaque constituted by echolucent and echogenic material in similar proportions (AbuRahma, Wulu et al. 2002). Petersen et al assessed the value of considering plaque heterogeneity as a predictor of death in hospitalised cardiological patients. They reported that of the 541 patients assessed, 361 had carotid plaques and during follow-up, 83 died (all-cause deaths). Using a multi-variable analysis model, they showed that plaque heterogeneity was an independent predictor of death and that patients with heterogeneous plaques had a Kaplan-Meier survival estimate of 73% compared with 90% for patients without plaque and 79% for patients with homogenous plaque ($p=0.0001$) (Petersen, Pecanha et al. 2006). Several hypotheses are raised by these findings. The presence of echolucent areas in an echogenic plaque may correspond to areas

of intra-plaque haemorrhage with increased risk of rupture and or thrombosis and therefore be associated with acute CVD events. Another explanation is that the presence of different components within the same plaque leads to opposing shear stress forces when the plaque is subjected to the stress of high velocity blood flow. This could increase the risk of rupture through mechanical stresses with the same end result: plaque rupture/ thrombosis and CVD acute events. Further studies assessing plaque mobility and its correlations with GSM and CVD events are required to answer these questions.

The relevance of considering plaque echogenicity stems from several studies which have highlighted the concept that in addition to the presence of plaque and its size, the texture or composition of plaque also correlates with the risk for CVD-related events. Many of these studies are undertaken in the context of cerebrovascular disease, correlating the findings of carotid US and recurrence of stroke. The general consensus is that the presence of echolucent plaques is associated with a significant increase of cerebral events compared with echogenic plaques (Mathiesen, Bonnaa et al. 2001, Biasi, Froio et al. 2004, Johnsen, Mathiesen et al. 2007, Kakkos, Stevens et al. 2007, Kakkos, Sabetai et al. 2009, Singh, Atam et al. 2013, Ariyoshi, Okuya et al. 2015). Overall, these studies have been done in either the general population or in specific risk groups, namely among diabetic patients and usually have a greater proportion of male subjects. Therefore, it may be difficult to transpose these findings to the setting of SLE where the prototypical patients are relatively young women with few traditional CVD-risk factors.

Some sub-group analysis however, focusing on specific subject groups may offer some insights. Reporting on the findings from the Tromsø study, Johnsen et al described how the presence of carotid plaque is a stronger predictor of MI in women compared with men (almost 3-fold increased risk). Moreover, it was found that the risk of MI among women increased with plaque echolucency, supporting the data previously discussed (Johnsen, Mathiesen et al. 2007). The vascular US cohort I tested included mainly women and for those who were found to have plaque, the median GSM was low, thus emphasizing how their risk for having a MI may be increased.

Although there appears to be some consensus that low GSM plaques are associated with increased prevalence of cerebrovascular events and overall CVD events, Zureik et al described, in a large study, (n=561) how aortic arterial stiffness correlates with the presence of echogenic plaques (qualitative scale score 3-4), regardless of the BP status and carotid IMT thickness (Zureik, Temmar et al. 2002, Zureik, Bureau et al. 2003). It is argued that both plaque echogenicity and arterial stiffness share common pathophysiological processes leading to connective tissue extracellular matrix deposition (collagen, proteoglycans and

elastin) and therefore, the presence of increased arterial stiffness could be expected in the presence of fibrous-rich and not lipid-rich plaques (Stary 1989, Stary, Chandler et al. 1995). Although the presence of lipid-rich plaques has been associated with increased plaque instability and risk of rupture, several cross-sectional studies report a strong association between the presence of increased arterial stiffening and CVD-related events, namely stroke, MI and congestive heart failure (Hirai, Sasayama et al. 1989, Meaume, Benetos et al. 2001, Meaume, Rudnichi et al. 2001, Mitchell, Tardif et al. 2001). The caveat is that there are no similar studies focusing on an SLE population and therefore the basis for extrapolating these findings to patients with Lupus is lacking.

Tarnoki et al published interesting data assessing the impact of the genetic background on the prevalence of plaque and its characteristics and concluded that there was a strong influence of heritability on the ultrasound characteristics of carotid plaque, arguing that unshared environmental factors account only for a modest portion of the variance (Tarnoki, Baracchini et al. 2012). There have been several studies discussing potential genes which may be implicated in the pathogenesis of SLE although a clear etiological link is yet to be firmly established. Whether there are genetic characteristics shared between SLE and plaque presence and its composition has not been described but this could be a potential link between these two apparently distinct clinical entities.

Plaque echogenicity may also be an important factor in the decision making process of whether or not to start lipid-lowering treatment in patients with SLE. Several groups have reported on the benefits of treatment with statins in regards to increasing plaque echogenicity even in non-hypercholesterolemic individuals, regardless of changes in plaque size or IMT thickness (Watanabe, Sugiyama et al. 2005, Kadoglou, Gerasimidis et al. 2008, Yamagishi, Kato et al. 2009). This raises the question of whether patients with SLE who are found to have plaque, particularly if it is echolucent, should be started on treatment with statins regardless of their lipid profile. To date, there is no specific guidance on this matter and the decision depends largely on the attending physician.

Regarding the factors that may influence plaque echogenicity, a myriad of potential biomarkers has been reported. Data from the Tromsø study has suggested that low HDL levels are independently associated with plaque echolucency (Mathiesen, Bonaa et al. 2001). Moreover, high HDL cholesterol levels appear to reduce plaque growth in subjects with pre-existing atherosclerosis, raising the hypothesis that HDL may in fact stabilize plaque and reduce lipid accumulation and intra-plaque inflammation (Johnsen, Mathiesen et al. 2005). There was no mention of HDL function assessment in this study, as only serum HDL concentration was determined. The Tromsø study also assessed 2726 post-menopausal

women and correlated bone density with plaque echogenicity, reporting that lower bone mass was associated with an increased risk of echogenic and even calcified atherosclerotic plaques (Jorgensen, Joakimsen et al. 2004). Finally, the influence of albuminuria, a known factor found to be associated with atherosclerosis, on plaque initiation and growth was assessed. Multivariate analysis supported the notion that higher albumin-to-creatinine ratio was associated with greater plaque area and increased plaque progression, particularly among patients who did not present plaque in the baseline assessment, leading to the conclusion that albuminuria is an independent risk factor for plaque initiation and growth (Jorgensen, Jenssen et al. 2007).

Increased BMI and other traditional lipid profile changes were also reported to correlate independently with increased plaque echolucency, namely, TG and IDL-cholesterol (Gronholdt, Nordestgaard et al. 1998). Diabetes was also found to be associated with increased plaque echolucency (Ostling, Hedblad et al. 2007) but in non-diabetic patients, high levels of glycated haemoglobin (HbA_{1c}) levels were independently associated with increased plaque echogenicity (Jorgensen, Jenssen et al. 2004). No clear explanation for this fact is offered, but it is hypothesised that the presence of raised HbA_{1c} may be associated with early atherosclerosis or that it may lead to accelerated atherosclerosis and consequently faster progression from echolucent to echogenic plaques. The Northern Manhattan Study reported on the influence of homocysteine in both tPA and plaque echogenicity and described that raised homocysteine levels were associated with increased tPA (Alsulaimani, Gardener et al. 2013). However, the influence of homocysteine in plaque texture was less clear as the top two quartiles of homocysteine were more likely to have either echolucent or echogenic plaque. The authors hypothesise that this U-shaped curve may signify that high homocysteine levels are a factor in both vulnerable echolucent plaques and in later echogenic plaques that may signify a longstanding atherosclerotic process.

Several groups have reported on the influence of inflammatory biomarkers and plaque echolucency, namely IL-6 (Yamagami, Kitagawa et al. 2004) and CRP (Gronholdt, Sillesen et al. 2001), but whether they are causal or merely accompany the atherosclerotic process is yet to be established.

In my vascular US cohort, I found that BP was weakly correlated with either TpA and TpT and this is probably due to the very low prevalence of hypertension among the patients assessed. Of the traditional CVD risk factors, I found that increased TG and homocysteine levels were associated with bigger plaques. Neither t-cholesterol nor LDL were found to correlate with plaque size and this is again probably due to the very low presence of dyslipidaemia in the patients tested. Nonetheless, the more sensitive t-cholesterol:HDL was

noted to positively correlate with both TpA and TpT. The presence of clinically active disease was associated with increased plaque burden but serological markers of disease activity were not, nor were any other auto-antibodies with the exception of anti-HDL IgG antibodies which were positively correlated with TpA and TpT as previously discussed.

The final point for discussion is the comparison between the vascular US findings between the SLE and the non-SLE cohorts. I would like to emphasise the limitations of this analysis. Firstly, there is the different demographic background: the SLE is a multi-ethnic, London-based cohort while the non-SLE is a Caucasian, Cypriot population with a specific genetic, social and environmental background. Secondly, age-matching was only possible for patients who were 40-year old or older (n=66) which further diminishes the size of the SLE sub-group included in the analysis. Thirdly, patients from the non-SLE cohort were not screened for established or likely CVD. Lastly, although the vascular US equipment, protocol and scanner (Dr Griffin) were the same for both cohorts, the assessment was not performed within the same timeframe nor was the physical circumstances similar as the non-SLE cohort was scanned in Cyprus. Therefore, any hypothesis raised by the comparison between these two groups must be cautious and viewed critically. Nonetheless, I believe that the data are sufficiently novel and relevant to be described, presented and discussed as it highlights a few interesting points.

There were no significant differences in CCA IMT between the two groups, and although the non-SLE cohort had slightly greater mean values, they were still well within the range of normal. However, patients with SLE had plaque in more territories than the non-SLE cohort and plaque area and thickness were significantly greater. Moreover, the plaques from patients with SLE had significantly higher mean GSM compared to non-SLE subjects. When comparing the characteristics from both groups, although by shared similar smoking and diabetes prevalence, non-SLE patients had an increased prevalence of high BP as well as higher t-cholesterol, LDL and TG levels and lower HDL. In contrast, patients with SLE had higher homocysteine levels. Higher serum creatinine level was also noted in the SLE cohort, but for the vast majority of patients it still fell within the normal range. However, a significant number of patients with SLE had increased urine protein-creatinine ratio and as previously discussed this may be associated with increased plaque initiation and growth. This suggests that although patients with SLE have fewer traditional CVD risk factors, they have increased atherosclerotic burden compared with non-SLE controls which is in keeping with all the literature regarding SLE-related mortality and morbidity.

Given that SLE is a chronic inflammatory disease, it could be argued that patients with Lupus would have more echolucent plaques compared with non-SLE controls and in fact, I

found a significant correlation between increased disease activity and TpA suggesting that increased inflammation is a relevant factor for atherogenesis in the context of SLE.

However, I found precisely the opposite: patients with SLE had significantly higher GSM compared with non-SLE controls. This could be due to several factors, none the least with the distinct demographic background of the two populations. Patients from the non-SLE cohort had a greater prevalence of a pro-atherogenic lipid profile and increased BP, which are two strong traditional CVD factors associated with increased plaque echolucency which could at least partially account for the lower GSM in this group. In addition, if we considered that the natural history of plaque is to become more echogenic over time (Stary 2000), it may be hypothesised that the reason why plaques are more echogenic in the SLE group is due to early plaque initiation. This seems to be plausible as shown on the regression model depicting age at scan plotted against TPT and TpA (Figure 25 and 26). Alternatively, it may be argued that the atherosclerotic process does not in fact start earlier in SLE, but may occur in an accelerated fashion which could also be supported by the later graphs. The fact that the growth pattern, i.e. the correlation between TpA and TpT and CCA IMT, is different in the SLE cohort compared with the non-SLE cohort supports the hypothesis that the atherosclerotic process is happening faster in SLE as there is very little IMT thickening even when plaque is present. This is supported by the fact that I found that patients with increased disease activity over time had increased GSM suggesting that in patients with active disease, the presence of accelerated atherosclerosis may be further enhanced.

The use of 3D US in the vascular US cohort added very little to what was found in the 2D assessment and this is likely to be due to the smaller number of plaques assessed by this method (n= 40) as only carotid plaques were considered and anatomical constraints rendered the technique impossible to perform.

5: FINAL DISCUSSION AND FUTURE WORK

Atherosclerosis has long ceased to be viewed as a rather passive cholesterol accumulation on the vascular walls which occurs inexorably over time. It is now understood as a dynamic accumulation of oxidized cholesterol over time that is primarily driven by the immune system (Libby, Ridker et al. 2002). The acknowledgement of the central role played by the immune system in the pathogenesis of atherosclerosis and consequently of cardiovascular disease, has led to an increased interest in the way atherosclerosis develops in the context of chronic inflammatory conditions. The conclusion is that autoimmunity and immune system dysfunction are associated with increased morbidity and mortality due to CVD. It is hypothesized that this could be due to accelerated atherosclerosis (Skaggs, Hahn et al. 2012). SLE in particular has become the paradigm of this concept, with several studies reporting a much increased risk of MI/ angina (Manzi, Meilahn et al. 1997), greater prevalence of carotid plaque in US assessment (Roman, Shanker et al. 2003), increased prevalence of coronary artery calcification on MRI assessment (Asanuma, Oeser et al. 2003) and overall increased CV risk independent of traditional risk factors (Esdaille, Abrahamowicz et al. 2001). Hence, SLE has been established as one of the strongest, independent risk factors for CVD (D'Cruz, Khamashta et al. 2007, Pennell and Keenan 2011).

Although several mechanisms have been proposed, the full pathogenesis which is likely to be multifactorial is yet to be fully established.

Nitric oxide metabolism has important implications in regulating endothelial function, with both protective and pathological properties. Given the associations between increased vascular activation and enhanced nitrosative stress, a plausible hypothesis would be that the chronic inflammation present in SLE, could lead to an increase NO release and consequently to increased concentration of nitrated proteins. The rationale which correlates these changes with increased atherosclerotic burden, would be that increased widespread vascular activation could lead to endothelial injury, one of the earliest phenomena in atherosclerosis. I chose to study three different targets for nitration: nucleosomes (NN), albumin (NA) and apoA1 (NapoA1). Although I did find an association between disease activity and NN, in particular with neuropsychiatric and vasculitic flares, no associations between levels of nitrated proteins with either IMT, presence of plaque, plaque size and echogenicity were evident. This finding suggests that the presence of serum protein nitration does not correlate with increased atherosclerotic burden in US assessment. However, this lack of correlation may be interpreted as a consequence of the protein targets I selected, or due to the fact that circulating nitrated proteins do not reach significantly high levels to elicit

significant changes and that the measurement of tissue nitrated proteins may be a more sensitive marker.

Another aspect that may be considered is the lipoprotein metabolism, particularly in what concerns HDL, whose atheroprotective role has long been established. ApoA1 is the main component of HDL and its integrity is essential to adequate HDL function. Reduced levels of ApoA1 are associated with increased CVD and the presence of increased nitrosative stress and nitrated Apo1 may be associated with impaired function and consequently, increased CV risk (Hahn 2010, O'Neill, Giles et al. 2010, Teixeira, Cutler et al. 2012). Patients with SLE have lower TApoA1 and higher nitrated ApoA1 than HC, regardless of disease activity, treatment or classical autoantibody profile (ENA, anti-dsDNA, C3).

A potential mechanism for the reduced TApoA1 levels in patients with SLE, is the presence of anti-ApoA1 antibodies reported in the serum of patients with SLE (Dinu, Merrill et al. 1998, Delgado Alves, Ames et al. 2002, Alves and Ames 2003, Delgado Alves, Kumar et al. 2003, Batuca, Ames et al. 2007, Batuca, Ames et al. 2009, O'Neill, Giles et al. 2010). I also found that anti-ApoA1 levels were significantly higher in SLE compared to healthy controls in all the groups studied. Almost one third of patients were positive for anti-apoA1 IgG very early in the disease course. However, I did not find any associations between anti-apoA1 IgG positivity early in disease and development of CVD later in life or increased atherosclerotic burden as assessed by vascular US. No significant associations between anti-ApoA1 and CVD-related variables were found. In contrast the presence of anti-HDL IgG in high titres did appear to be associated with lower TApoA1 levels. This association supports the hypothesis that the presence of anti-HDL antibodies might be associated with increased ApoA1 clearance, possibly due to the formation of immune complexes, which would then support the notion that these antibodies may contribute to the increased CVD risk found in SLE. The presence of high levels of nitrated ApoA1 may also be a mechanism of HDL dysfunction and it would be relevant to assess the correlation and potentially causal association between these two variables. The occurrence of dysfunctional HDL in inflammatory diseases, such as SLE, has also been suggested as a potentially important factor in the increased CVD risk observed in these patients (Hahn, Grossman et al. 2007). The presence of nitrated ApoA1 could be a potential mechanism for impaired HDL function.

The challenge for the clinician is to identify patients with greater atherosclerotic burden at a very early stage, preferably before symptomatic CVD emerges. Although vascular US is a reliable and reproducible method, it is not readily available and the plaque texture characterization is not performed routinely by most imaging departments. Therefore, the

ideal situation would be to identify a panel of biomarkers which correlated with the presence of atherosclerotic disease.

The use of vascular US, in particular of multiple vascular territories (central/ carotid and peripheral/ femorals) is a non-invasive, non-irradiating, reproducible method that allows for an accurate assessment of the true atherosclerotic burden of an otherwise asymptomatic patient. Moreover, the extent of atherosclerotic disease (i.e. presence of plaque in multiple sites) has prognostic value in predicting the risk of coronary events (Schiano, Sirico et al. 2012). The global atherosclerotic load may be expressed as the total plaque area (TpA) and/ or total plaque thickness (TpT).

In the SLE cohort I selected, 36 out of 100 patients with SLE with no evidence of CVD already had plaque which falls into the range of what has been previously reported (Manzi, Selzer et al. 1999, Svenungsson, Jensen-Urstad et al. 2001, Doria, Shoenfeld et al. 2003, Roman, Shanker et al. 2003, Selzer, Sutton-Tyrrell et al. 2004, Wolak, Todosoui et al. 2004). The much higher prevalence of atherosclerosis among individuals who typically have a low score in the traditional risk stratification tools (Bessant, Hingorani et al. 2004, O'Neill, Pego-Reigosa et al. 2009) comparably with matched HC (Svenungsson, Jensen-Urstad et al. 2001, Roman, Shanker et al. 2003, Thompson, Sutton-Tyrrell et al. 2008) may be potentiated by the difficulty in diagnosing subclinical CVD and establishing primary prevention strategies.

I attempted to combine the vascular US findings obtained from a cohort of patients with SLE with variables measured routinely in clinical practice (BP, CRP, renal function, ESR), some tests which can be performed in most hospitals (homocysteine, vitamin D) and with specific research biomarkers (anti-ApoA1, anti-HDL, nitrated proteins). I found that the BP was weakly correlated with both TpA and TpT and this is probably due to the very low prevalence of hypertension among the patients assessed. Among the other traditional CVD risk factors, I found that increased TG and homocysteine levels were associated with increased plaque size. Neither t-cholesterol nor LDL were found to correlate with plaque size and this is again probably due to the very low presence of dyslipidaemia in the patients tested. Nonetheless, the more sensitive t-cholesterol:HDL was noted to correlate positively with both TpA and TpT. The presence of clinically active disease was associated with increased plaque burden, but serological markers of disease activity were not, nor were any other auto-antibodies with the exception of anti-HDL IgG antibodies which were positively correlated with TpA and TpT. Finally, I found a significant correlation between increased disease activity and TpA suggesting that increased inflammation may be a relevant factor for atherogenesis in the context of SLE. Patients with increased disease activity over time also had increased GSM

suggesting that in patients with active disease, plaque progression may be developing differently as discussed below.

It is worth comparing the vascular US findings between the SLE and the non-SLE cohorts. Despite the limitations previously noted, this analysis raises a few relevant points. The first is not new as it concerns the much greater atherosclerotic burden of patients with SLE compared with age and sex-matched controls, as expressed by the differences regarding TpA, TpT and number of vascular territories involved. The emphasis of these findings is further enhanced by the fact that patients with SLE had fewer traditional CVD risk factors compared with the non-SLE subjects, particularly in regards to the lipid and BP profile.

The second point regards plaque echogenicity. I found that although the majority of plaques identified in the SLE cohort had a low GSM (mean GSM 48) and could therefore be considered to be predominately echolucent or lipid rich (Goncalves, Lindholm et al. 2004, Sztajzel, Momjian et al. 2005), non-SLE individuals had significantly lower GSM. The association between plaque echogenicity and risk of CVD-events has not been established unequivocally. Although the general consensus is that the presence of echolucent plaques is associated with increased cerebral events (Mathiesen, Bonnaa et al. 2001, Biasi, Froio et al. 2004, Johnsen, Mathiesen et al. 2007, Kakkos, Stevens et al. 2007, Kakkos, Sabetai et al. 2009, Singh, Atam et al. 2013, Ariyoshi, Okuya et al. 2015), their presence has been shown to correlate with aortic stiffness (Zureik, Temmar et al. 2002, Zureik, Bureau et al. 2003) which in turn has been associated with CVD-related events, namely stroke, MI and congestive heart failure (Hirai, Sasayama et al. 1989, Meaume, Benetos et al. 2001, Meaume, Rudnichi et al. 2001, Mitchell, Tardif et al. 2001). Overall, these studies have been done in either the general population or in specific risk groups, namely among diabetic patients and usually have a greater proportion of male subjects. Therefore, it may be difficult to compare these findings to the setting of SLE where in the main the patients are relatively young women with few traditional CVD-risk factors.

Several explanations for the differences found between the SLE and the non-SLE cohorts may be hypothesised ranging from the genetic differences of two ethnically different populations to the differences in lipid (pro-atherogenic in the non-SLE cohort) and BP profile (higher in the non-SLE group).

An alternative explanation for why the plaques found in patients with SLE are more echogenic than those found on non-SLE individuals could be their “age”. If we accept that “older” plaques are more echogenic (Stary 2000), the reason why patients with SLE have plaques with increased GSM compared with non-SLE individuals may be that their plaque

formation starts earlier. Alternatively the atherosclerotic process may start at a similar age, but occurs in an accelerated fashion. Both these hypotheses are supported by the regression models depicting age at scan plotted against TPT and TpA. The later hypothesis that the crucial difference is the rate of plaque progression is further supported by the fact that the plaque growth pattern (i.e. the correlation between TpA and TpT and CCA IMT) is different in the SLE cohort compared with the non-SLE cohort. This observation suggests that the atherosclerotic process is happening faster in SLE as there is very little IMT thickening even when plaque is present. This could be the reason why patients with sustained active disease have more echogenic plaques, as the presence of chronic inflammation could further enhance a process of accelerated atherosclerosis.

In this final chapter of my thesis, I think it is appropriate to reflect on the work I have done so far, highlight the questions it raised and consider how they may be pursued in the future.

One of the main achievements stemming from this thesis was the construction of a comprehensive database for 100 patients with SLE which includes full demographic and SLE-related characterization, notably disease course, main organs or systems involved, immunological profile, disease activity over time and type of treatment as well as CVD-specific risk factors, namely lipid profile, smoking habits and BMI. This is a useful data set to build upon the work I have done and explore further the potential implications of other variables which can potentially influence the increased atherosclerotic burden of patients with SLE.

Ideally, a follow-up project to re-assess these same patients in 2-3 years' time will be developed. This would allow comparing plaque and IMT progression, not only in terms of qualitative measures such as IMT/ plaque thickness and area, but also plaque texture modifications over time.

With regards to assessing other serological variables which might correlate with plaque characteristics, I would like to measure dysfunctional HDL levels in these patients and see whether any significant associations emerge. I would also like to investigate whether the presence of anti-ApoA1 IgG/ IgM and anti-HDL antibodies correlates with increased dysfunctional HDL.

Moreover, I think it would be interesting to explore different targets which may correlate better with vascular damage and therefore be associated with subclinical atherosclerosis. A potential candidate would be endothelial microparticles, as it has been suggested that they

are increased in SLE and other systemic inflammatory conditions. For this project, plasma samples rather than sera would be necessary and I have already collected plasma samples for a majority of patients.

Finally, at present, a motion analysis software is being developed in order to allow for a quantification of arterial expansion and eventually correlate it with other, more established methods such as pulse wave velocity. The goal is to find an US-based surrogate marker that allows for a non-invasive, reproducible method to assess arterial stiffness. I am also collaborating with the developers of this software with the goal to find a measurable variable that translates the movement of plaque observed with every cardiac cycle. This is being accomplished by identifying the different motion vectors generated by the movement of plaque with systolic and diastolic variations. The hypothesis is that if, within a plaque, opposite motion vectors are identified (discordant plaque motion), it may signify that there are increased shear stress forces which may then be associated with increased risk of plaque rupture (i.e. unstable/ vulnerable plaque). The opposite could also be found to be true, i.e. plaques which have homogenous motion vectors may have a reduced risk of rupture and therefore be more stable (concordant plaque motion). Should this software prove to be reliable and yield a quantitative variable, associations with other biological variables as the ones I have explored in my thesis may be identified. Another goal would be to design a prospective study to assess whether the presence of distinctive plaque and arterial wall motion patterns influences prognosis, specifically whether it is associated with differences in the incidence of CVD-related events. I have already obtained the required imaging data, including arterial motion loops and plaque motion loops, and am working with Professor Nicolaides and Dr Griffin with a view to continue to develop this potentially new tool.

Overall, my goal would be to try to establish a reliable and accurate risk profile of patients with SLE who are more likely to have subclinical atherosclerotic disease with the intention of obtaining a vascular US-based risk assessment thus allowing for more aggressive SLE treatment as well as for stricter management of other aspects such as lipid profile, hypertension and diabetes. Ultimately I would like to have contributed to a better assessment of the actual atherosclerotic burden of patients with SLE thereby allowing primary prevention strategies to be developed which will minimize the occurrence of CVD-related events.

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Review Article

Imaging Assessment of Cardiovascular Disease in Systemic Lupus Erythematosus

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Systemic lupus erythematosus is a multisystem, autoimmune disease known to be one of the strongest risk factors for atherosclerosis. Patients with SLE have an excess cardiovascular risk compared with the general population, leading to increased cardiovascular morbidity and mortality. Although the precise explanation for this is yet to be established, it seems to be associated with the presence of an accelerated atherosclerotic process, arising from the combination of traditional and lupus-specific risk factors. Moreover, cardiovascular-disease associated mortality in patients with SLE has not improved over time. One of the main reasons for this is the poor performance of standard risk stratification tools on assessing the cardiovascular risk of patients with SLE. Therefore, establishing alternative ways to identify patients at increased risk efficiently is essential. With recent developments in several imaging techniques, the ultimate goal of cardiovascular assessment will shift from assessing symptomatic patients to diagnosing early cardiovascular disease in asymptomatic patients which will hopefully help us to prevent its progression. This review will focus on the current status of the imaging tools available to assess cardiac and vascular function in patients with SLE.

1. Introduction

Systemic lupus erythematosus (SLE) is a multisystem, autoimmune disease and is one of the strongest known risk factors for atherosclerosis and coronary artery disease (CAD) [1, 2]. The range of cardiovascular disease (CVD) in SLE is broad and includes atherosclerosis, vascular inflammation, Raynaud's phenomenon, endothelial dysfunction, and a pro-coagulant tendency associated with antiphospholipid antibodies. The impact of SLE-associated CVD on both mortality and morbidity is impressive: the incidence of CAD is over 7 times greater in patients with SLE than in healthy controls, even when matched for cardiovascular risk factors [3]. Moreover, female patients with SLE between 35 and 44 years old have an incidence of myocardial infarction over 50 times greater than the observed in the Framingham dataset [4]. These findings account for the bimodal mortality pattern in SLE: an early peak (<1 year of diagnosis) associated with renal involvement and infection and a later peak (8 years after diagnosis) due to premature myocardial infarction [5]. In addition to an increased risk of CAD, patients with SLE

are at greater risk for stroke, with a prevalence that can reach 20% and with a high recurrence rate and greater mortality than matched controls [6].

The reason why patients with SLE have a higher cardiovascular mortality and morbidity seems to be related to the presence of an accelerated atherosclerotic process [7, 8], which seems to be due to a complex interplay of traditional and lupus-specific risk factors [3, 9–11]. On the one hand, some of the factors contributing to an accelerated atherosclerosis may be associated with the disease itself: the systemic inflammation associated with poorly controlled SLE could contribute to plaque destabilization. On the other hand, patients with SLE have a high prevalence of traditional CVD risk factors [3, 10] such as hypertension, altered lipid profile [12], and impaired glucose tolerance, which to some extent result from chronic corticosteroid therapy [13]. However, not only has no unequivocal correlation been established between corticosteroid use and atherosclerosis in SLE, but some evidence seems to suggest an increased cardiovascular risk among patients who are under treated with steroids, thus implying that having poorer disease control is associated

with a higher vascular risk than steroid therapy *per se* [7]. The generally accepted notion is that systemic inflammation related to SLE contributes both to an accelerated atherosclerosis and plaque destabilization which in turn is the major cause of acute plaque disruption responsible for acute cardiovascular events such as myocardial infarction.

The relevance of accurate cardiovascular assessment in patients with SLE has been emphasized by recent studies that show that mortality associated with CVD has not improved over time, opposing the trend seen for other causes of mortality such as lupus nephritis [14, 15]. Several reasons can be hypothesised to explain this but one of the strongest is the poor performance of standard risk stratification tools (based on the Framingham risk equation) [16] in patients with SLE, which prevents an accurate assessment of the actual cardiovascular risk of the individual patient [9, 17]. It is, therefore, essential to find alternative ways to assess and identify patients with SLE at increased risk for CVD efficiently. Several imaging techniques have been studied as potential tools to assess these patients better, with particular emphasis in noninvasive screening tools aimed at detecting subclinical atherosclerosis.

This paper will focus on the current status of imaging assessment of cardiac and vascular function among patients with SLE. There are two possible roles for this type of assessment. One is to identify CVD in patients with suspicious symptoms or other good reasons to suspect CVD (e.g., heavy smoking). The more challenging role is to diagnose CVD in asymptomatic patients with few or no risk factors other than SLE itself. It is important to remember that though SLE is associated with an increased relative risk of developing CVD compared to healthy controls, the absolute risk of developing CVD in an individual patient remains small. For example, in a recent multicentre study of 1249 patients recruited within 15 months of the diagnosis of SLE and followed for up to 8 years, only 74 patients developed CVD [18]. Thus, it would be difficult to justify invasive or repeated imaging to screen for CVD in the majority of patients with SLE.

2. Assessing Cardiac Involvement

SLE-associated cardiac involvement can be divided into 4 groups: pericarditis/pericardial effusion, valvular disease, myocardial dysfunction, and coronary-artery disease (CAD). For the purpose of this paper, we will focus only on the last two groups.

2.1. SLE-Associated Myocardial Dysfunction. In SLE, myocardial dysfunction may be due to several features such as CAD, valvular disease, drug-related cardiotoxicity (e.g., cyclophosphamide and chloroquine), and lupus myocarditis. After the introduction of corticosteroid therapy, the prevalence of autopsy-identified SLE-related myocarditis decreased from 50%–75% [19] to 25%–30% [20]. However, clinically evident lupus myocarditis is identified in less than 10% of patients, showing the high prevalence of subclinical disease [21]. In fact, clinical manifestations of SLE-associated myocarditis are subtle and nonspecific. The fact that systolic

function is preserved until late stages of the disease accounts for the low sensitivity of echocardiographic assessment [22]. Although still considered the gold standard for pericardial and valvular evaluation [22, 23], its use in lupus myocarditis diagnosis is limited. However, it can give some indication of left ventricle diastolic dysfunction through the presence of impaired myocardial relaxation, decreased compliance, and increased filling pressure [22]. Another way to assess cardiac function is through left ventricle angiography, both by the conventional method and by using Technetium-99m myocardial perfusion imaging (SPECT), which permits accurate assessment of left ventricle volume and function [24, 25]. However, these methods have largely been replaced by MRI imaging [26].

The definite diagnosis of lupus myocarditis is histological, with typical features being interstitial oedema, focal necrosis/fibrosis, and focal or diffuse inflammatory cellular infiltrates [27]. However, despite being the gold standard for diagnosis, endomyocardial biopsy cannot be used routinely or repeatedly, particularly in asymptomatic patients. Cardiac magnetic resonance (CMR) is sensitive to many of the changes that characterize lupus myocarditis, particularly through T2-weighted imaging (myocardial oedema) [28, 29] and early (EGE) and late (LGE) gadolinium-enhanced CMR [28, 30]. The combination of EGE, LGE, and T2 imaging sequences has been reported to have 76% sensitivity and 95.5% specificity for the detection of myocardial inflammation [28]. In addition, CMR is superior to other techniques in assessment of left ventricle size, function, and mass, provides high spatial resolution, is noninvasive and has high reproducibility and low intra and interobserver variability [26]. In a recent study, we carried out CMR and transthoracic echocardiography in 22 patients with SLE (11 patients with previous CVD and 11 age-sex matched controls) [30]. We found that CMR was more sensitive than echocardiography for the detection of myocardial changes, especially late gadolinium enhancement (LGE) in areas of previous infarction [30]. In contrast to a previous report [31], we did not find widespread small areas of LGE in the myocardial tissues of these patients. Mavrogeni et al. [32] reported LGE in 18/20 patients with autoimmune rheumatic diseases (three with SLE). Ten patients also had myocardial biopsies with a 50% agreement between biopsy and CMR results.

CT imaging is not considered an adequate tool for evaluation of cardiac muscle due to radiation exposure, movement artefacts and application of contrast media which prevents use in patients with renal failure and severe heart failure [33].

2.2. Coronary Artery Assessment. As stated before, SLE is associated with a significantly increased risk of CAD. The presence of CAD can be evaluated directly by coronary arteriography and indirectly by assessing left ventricle ejection function and ventricular wall motion through radionuclide ventriculography, echocardiography, SPECT, and CMR [22].

2D echocardiography is the most widely used method for routine assessment of left ventricle ejection fraction in patients with known CAD. Other methods, such as tissue Doppler imaging and 3D echocardiography have been proposed

as superior alternatives; however, they still have not replaced conventional echocardiography [22]. Turiel et al. [34] have proposed a global index of left ventricle function (TEI index) aimed at systolic and diastolic left ventricle function. However, its validity in SLE has yet to be shown. Stress echocardiography using either exercise or pharmacological stimulus can be a useful method for diagnosis and risk stratification in patients with suspected or known CAD [22].

Presently, several studies have shown the utility of MRI imaging in assessment of CAD—though not in patients with SLE [26, 28, 35]. Stress CMR (i.e., using dobutamine or adenosine) is an accurate method to identify ischemia-induced wall motion abnormalities, with a greater sensitivity (86% versus 74%) and specificity (86% versus 70%) than stress echocardiography [36]. In addition, perfusion defects can be identified with gadolinium-enhanced CMRI as well as positron emission tomography (PET) [37] and SPECT [25]. One additional benefit from PET imaging is the possibility of identifying stable plaques as a high uptake of contrast seems to be associated with a higher macrophage content which would correlate with the presence of intraplaque active inflammation [38]. However, the use of radioisotopes for PET limits its applicability.

Electron beam CT (EB-CT) can be used to quantify coronary artery calcification as a measure of coronary atherosclerosis. Asanuma et al. [7] compared EB-CT findings in 65 patients with SLE and 68 age-/sex-/ethnicity-matched controls. Mean calcification scores was significantly higher in patients than controls. After adjustment for cardiac risk factors including age, sex, smoking, hypertension, triglyceride, and homocysteine levels, patients with SLE were still 9.8 times more likely to have coronary calcification than controls. The reason for this was unclear. In a subsequent paper Kiani et al. [39] found coronary calcification in 43% of 200 women with SLE, but the only factors predicting this in multiple logistic regression analysis were age and body mass index. SLE disease activity was not associated with coronary calcification. CT angiography can be used to detect plaques in the coronary arteries. In a recent study [40], Ishimori et al. carried out both adenosine stress CMR and CTA in 18 female patients with SLE who had suffered chest pain within the previous six months and in 10 healthy control women. Eight patients with SLE, but no control subjects had abnormal perfusion on stress CMR. This was severe in 7 cases even though none of the patients had obstructive CAD detectable by CTA and only two of the 18 subjects had any CTA abnormalities. The perfusion defects were not characteristic of coronary artery disease [40]. Thus, it seems likely that stress CMR was detecting microvascular ischemia in patients with SLE though larger studies are required.

Invasive methods for assessing coronary circulation such as intravascular ultrasound (IVUS) and IVUS with virtual histology [41], optical coherence tomography [42], coronary angiography [43], and invasive MRI [44] may prove their usefulness in the future by allowing direct plaque imaging. However, presently their predictive value and impact on risk stratification is yet to be established.

3. Assessing Peripheral Vascular Involvement

Peripheral vascular involvement in SLE can be associated with active vasculitis, endothelial dysfunction and atherosclerosis. For the purpose of this paper, we will focus on the latter two.

3.1. Endothelial Dysfunction. The endothelium is the main regulator of vascular wall homeostasis. It regulates vascular tone and permeability, platelet and leukocyte adhesion and aggregation, and finally, vascular thrombosis. The term “endothelial dysfunction” describes a nonadaptive state of phenotypic modulation characterized by a loss or deregulation of the homeostatic mechanisms operative in healthy endothelial cells [45]. Current evidence suggests that endothelial dysfunction is an early event in atherogenesis and contributes to all the stages of plaque development [46]. Although there are currently no imaging methods that can effectively assess endothelial function, several functional methods have been developed to try and address this issue. The hallmark of endothelial dysfunction is an impaired endothelium-dependent vasodilatation [47]. Peripheral studies include flow-mediated vasodilatation assessment [47–49], forearm perfusion techniques, pulse wave analysis, and skin laser Doppler flowmetry [45]. Other potential markers of endothelial dysfunction correlate with circulating procoagulant, prothrombotic, and proinflammatory mediators, but with the exception of C-reactive protein, evidence for their independent predictive value is still lacking [45]. Although very few studies of this nature have been done in SLE [48, 49], attenuated flow-mediated dilation has been a consistent finding, suggesting the presence of impaired endothelial function in these patients even before overt cardiovascular disease is apparent.

3.2. Peripheral Vascular Assessment. The presence of common carotid artery intimal-medial thickening and discrete, nonobstructive carotid atherosclerosis has been shown to be independently associated with subsequent cardiovascular risk in several longitudinal studies [50, 51].

Ultrasound assessment of carotid atherosclerosis is an accurate, noninvasive method that allows for assessment of arterial wall thickness and degree of plaque. Manzi et al. [52] studied the prevalence of carotid atherosclerosis as measured by B-mode ultrasound in 175 women with SLE, finding that 40% had at least 1 focal plaque and that more than 20% had a at least one large plaque (>50% of the vessel diameter) or multiple plaques with at least one medium plaque (30%–50% of the vessel diameter). Patients with higher cumulative damage measured by the modified Systemic Lupus International Collaborative Clinics (SLICC) damage score were more likely to have plaque, even after excluding the cardiovascular components of the SLICC index. A strong association between duration of use and cumulative dose of corticosteroids was also found. Other groups, working independently have also found a prevalence of carotid plaque in the order of 40% in patients with SLE [8]. A longitudinal study from the Manzi group [53] assessed plaque progression in 217 female patients with SLE followed for 10 years using

TABLE 1: Overview and comparison of different imaging methods in atherosclerotic plaque assessment (IMT: intima-media thickness; CVD: cardiovascular disease).

Imaging method	Plaque characterization	Advantages	Disadvantages	Published data from patients with SLE
Carotid ultrasound	IMT and plaque in carotid arteries	No radiation rapid-convenient correlates with risk of future CVD	Interpretation is operator dependent. High frequency of plaque in Patients with SLE (clinical implications unclear) Expensive	Yes [22, 23, 54, 55, 58]
Magnetic resonance imaging (MRI)	Structure of myocardium quantification of lipid content	No radiation more sensitive than echo for myocardial change	use of gadolinium limited in patients with renal impairment motion artefacts. Lower spatial resolution in vascular assessment. Longer length of study time	Yes [2, 13, 18, 30, 33–36]
Computed tomography (CT)	Quantification of calcium, fibrous and lipid component	Noninvasive detection of vulnerable plaques	Motion artefacts. Contraindicated in renal impairment Low resolution	Yes [17, 41, 42]
Intravascular ultrasound-based methods	Plaque volume Luminal and vessel dimensions calcium content	Good penetration depth complements coronary angiography	Invasive lower spatial resolution	No
Positron emission tomography (PET)	Plaque macrophage content		Not established for widespread clinical use	Yes [26, 27]
Optical CT	Plaque microstructure (fibrous cap thickness measurement)	High spatial resolution	Invasive limited depth of penetration	No
Invasive MR	Plaque morphology and structure		Not established for widespread clinical use	No
Coronary angioscopy	Direct plaque surface visualization	Three-dimensional view of plaque	Superficial assessment of plaque. Risk of coronary occlusion	No

ultrasound. Progression of plaque occurred in 27% of patients and, overall, the mean increase in intima-media thickness was 0.011 mm/year. Plaque progression was greater in patients with SLE when compared with matched controls, suggesting that B-mode ultrasound may be a useful surrogate end point in SLE clinical management [53]. Importantly, this group went on to show, for the first time, that increase in IMT or the presence of plaque predicted increased risk of cardiovascular events [54]. They followed 224 women with lupus but no previous cardiovascular events. Over a 10-year followup period, 73 of them suffered either cardiac or cerebrovascular events. In multivariable analysis, higher IMT and presence of plaque at baseline predicted increased risk of cardiovascular events. For IMT, the hazard ratio was 1.24 (95% CI 1.04 to 1.48) per mm increase and the hazard ratio for presence of plaque was 5.97 (95% CI 1.52 to 23.38) [54]. Further enhancement of ultrasound assessment of carotid plaques can be achieved using integrated backscatter analysis of carotid-intima complex. This method has been shown to correlate with the calcium and collagen content of vascular wall, therefore noninvasively evaluating arterial sclerosis [55]. However, its usefulness in SLE has not been established [56].

High-resolution CT imaging has also been studied as an alternative and more accurate method of assessing carotid plaques. CT angiography has been shown to not only provide an accurate analysis of the degree of stenosis but also to correlate with histological findings of atheromatous plaques at the carotid bifurcation [57]. However, limitations associated with the use of contrast and radiation exposure are of concern.

A potential role for MRI imaging has emerged; as its use has much less limitation than CT methods [58], and there is a good correlation between them. In addition to assessing intimal and medial thickening, both methods yield information concerning the pattern of plaque calcification. Whether this correlates with the risk of embolic stroke is yet to be definitely established.

Presently, cutting edge, multimodal imaging research using animal models is aimed at determining ways to accurately assess plaque stability [59–61]. In animal models, factors like atherosclerotic plaque neovascularisation, thickness of fibrous plaque, lipid-rich necrotic-core, and macrophage content have been related to an increased plaque disruption risk. However, clinical implications in humans have not been established.

Table 1 summarises the different methods of imaging described in this paper with their advantages and disadvantages.

4. Conclusion

In summary, patients with SLE have a high risk of developing CVD. Despite their relevance, traditional reversible risk factors solely cannot account for the overall cardiovascular risk increase, which also depends on disease and treatment related issues. In this paper, we have described a number of technological advances that have enhanced the ability of clinicians to assess the myocardium, coronary arteries, and peripheral vessels in patients with CVD. For most of them, there is little or no information about use in patients with SLE. Some of these imaging techniques, for example, PET scanning and CT angiography, should clearly be reserved for patients with SLE with known CVD or very high CVD risk (based on traditional risk factors as well as the presence of SLE). Others, such as echocardiography and carotid ultrasound are convenient and noninvasive and could be used as screening tools in asymptomatic patients though it is still unclear how best to manage patients who have abnormalities on these tests. Perhaps the best way to use these imaging methods in the future will be in combination with assessment of traditional risk factors, disease activity measurements, and blood tests relevant to CVD [62]. This holistic assessment could then be used to identify patients who would benefit from more accurate but more invasive imaging methods such as cardiac MRI.

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RESEARCH ARTICLE

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Serum nitrated nucleosome levels in patients with systemic lupus erythematosus: a retrospective longitudinal cohort study

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Abstract

Introduction: Circulating nucleosomes released from apoptotic cells are important in the pathogenesis of systemic lupus erythematosus (SLE). Both nucleosomes and anti-nucleosome antibodies are deposited in inflamed tissues in patients with SLE. Active inflammation promotes nitration of tyrosine residues on serum proteins. Our hypothesis was that levels of nitrated nucleosomes would be elevated in patients with SLE and could be associated with disease activity. We therefore carried out a retrospective longitudinal study to investigate factors affecting levels of nitrated nucleosomes (NN) in patients with SLE.

Methods: A novel serum ELISA was developed to measure serum NN and modified to measure serum nitrated albumin (NA). Levels of both NN and NA were measured in 397 samples from 49 patients with SLE followed through periods of disease flare and remission for a mean of 89 months. Anti-nucleosome antibody (anti-nuc) levels were measured in the same samples. The effects of 24 different clinical, demographic and serological variables on NN, NA and anti-nuc levels were assessed by univariable and multivariable analysis.

Results: Patients with SLE had higher mean NN than healthy controls or patients with other autoimmune rheumatic diseases ($P=0.01$). Serum samples from 18 out of 49 (36.7%) of SLE patients were never positive for NN. This group of 18 patients was characterized by lower anti-double stranded DNA antibodies (anti-dsDNA), disease activity and use of immunosuppressants. In the remaining 63.3%, NN levels were variable. High NN was significantly associated with anti-Sm antibodies, vasculitis, immunosuppressants, hydroxychloroquine and age at diagnosis. NN levels were raised in neuropsychiatric flares. NN levels did not completely parallel NA results, thus providing additional information over measuring nitration status alone. NN levels were not associated with anti-nuc levels.

Conclusions: NN are raised in a subset of patients with SLE, particularly those who are anti-Sm positive. Elevated NN may be a marker of vascular activation and neuropsychiatric flares in these patients.

Introduction

Nitration and nucleosomes are both relevant to the pathogenesis of systemic lupus erythematosus (SLE). Nitration may be linked to development of cardiovascular disease (CVD). Patients with SLE have an increased risk of developing CVD [1] for reasons that are not fully understood [2,3]. Nitric oxide (NO) produced by the vascular endothelium is an important metabolite involved in processes

such as vasodilatation and inhibition of platelet aggregation [4]. When produced in excess, for example under conditions of systemic inflammation, NO can cause chemical alteration of lipids and proteins. In particular, tyrosine residues within proteins can be nitrated irreversibly, forming nitrotyrosine. In patients with SLE, the serum nitrite level (an index for NO production) correlates with disease activity and levels of anti-double stranded DNA (anti-dsDNA) antibodies [4]. Patients with active lupus nephritis have higher levels of nitrotyrosine than those without renal disease [5,6]. In theory, any serum protein containing tyrosine residues may be nitrated. We are particularly interested in nitration of histones within nucleosomes.

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Nucleosomes are released during apoptosis and this apoptotic debris is not cleared efficiently in patients with SLE [7]. Both nucleosome and anti-nucleosome antibody levels are elevated in these patients [8] and deposition of nucleosome-anti-nucleosome complexes is important in lupus nephritis [9]. Thus, our hypothesis is that levels of nitrated nucleosomes (NN) in the serum of patients with SLE could rise, particularly during flares of disease activity. We developed a novel enzyme-linked immunosorbent assay (ELISA) to test this hypothesis.

The principle of this novel ELISA is that serum proteins containing nitrotyrosine residues are captured on the plate using an anti-nitrotyrosine antibody and the subset of captured proteins that contain histones are then detected using a polyclonal anti-histone antibody. The method detects any analyte that contains both histones and nitrotyrosine. Since histones in serum occur primarily in the form of nucleosomes we refer to this as a NN ELISA rather than nitrated histone ELISA. Nucleosomes nitrated on proteins other than histones would also be detected. Hence, the ELISA detects the presence of nitrated tyrosine residues upon a protein either complexed with histones or upon core histones themselves.

We carried out measurements of NN levels in 397 samples taken from 49 patients at different time points, including times of disease flare and remission. We carried out univariable and multivariable analyses to determine the demographic and clinical factors that are associated with NN levels. We also measured levels of nitrated albumin (NA) as a surrogate marker of overall nitrative stress (given that albumin is a ubiquitous protein in serum) and the levels of anti-nucleosome antibodies (anti-nuc). Anti-nuc levels were measured to test whether they correlate with NN levels, which could be the case if nitration of nucleosomes is important in making them more antigenic.

Methods

Patients and samples

Longitudinal serum samples ($n = 397$) were selected retrospectively from a cohort of 49 patients with SLE followed at University College London Hospital (UCLH) with a mean of eight samples per patient (SD 2.16; min 3; max 14) and a mean follow-up of 89 months (SD 46; min 14; max 180). All patients fulfilled the revised American College of Rheumatology (ACR) classification criteria for SLE [10]. We particularly selected patients who had suffered flares of disease activity. Samples were also obtained from 37 healthy control subjects and 38 autoimmune disease controls (13 with rheumatoid arthritis, 12 with myositis and 13 with Sjogren's syndrome).

For all SLE patient samples where data were available (94% of samples), we obtained anti-dsDNA antibody and complement C3 levels and disease activity from the date of the sample and from the previous three assessments.

Anti-dsDNA antibody and C3 levels were measured in the routine clinical laboratory at UCLH using enzyme-linked immunosorbent assay (ELISA) (Shield Diagnostics, Dundee, UK) and laser nephelometry, respectively. Based on the normal limits for our laboratory, anti-dsDNA level >50 IU/ml was defined as high and C3 level <0.9 g/l as low.

Disease activity was measured using the classic British Isles Lupus Assessment Group (BILAG) index [11]. The more recent BILAG 2004 index was not used as many of the samples had been taken before 2004. Current activity (on the date of the sample) for each system was defined as high if the BILAG score was A or B and low if it was C, D or E. Disease activity over the most recent four assessments was characterized as persistently low activity (if BILAG C, D or E was recorded in all systems on each occasion) or persistently moderate-high activity (in the presence of A or ≥ 1 B score in any BILAG system on at least two out of four occasions). Over 90% of all samples fell into one of those two categories and the rest were excluded from this part of the analysis.

Data on ethnicity, gender, drug therapy and anti-Sm, anti-RNP, anti-Ro and anti-La (all tested by ELISA) antibody status of the patients were obtained from the clinical records of the patients.

Ethical approval was granted by the joint UCL/UCLH Research Ethics Committee and subjects gave informed consent for use of their stored serum samples.

Serum assays

Capture ELISA to detect nitrated nucleosomes

The whole assay was done at room temperature (RT) except where specified and plates were washed three to four times with PBS-0.1% Tween (PBST) between steps. We divided 96-well streptavidin plates in half: the test side was coated with biotinylated polyclonal goat anti-nitrotyrosine antibody (Abcam 27646, VWR Lutterworth, UK) diluted 1:1,000 in PBS and the control side coated with PBS (75 μ L per well). After one hour incubation, plates were washed and blocked with 200 μ L of 0.5% ovalbumin in PBST (OVA-BST) for one hour. After washing, serum samples were loaded in duplicate onto the plates (100 μ L/well) at 1:30 dilution in PBS such that each sample was loaded in two wells on the test side and two matching wells on the control side. After one hour incubation at 37°C and washing, 50 μ L per well of rabbit anti-histone H3 antibody (sc-10809, Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted at 1:2,000 in OVA-BST were added and the plates were incubated for one hour. After washing, 50 μ L per well of goat anti-rabbit IgG horse radish peroxidase (HRP) conjugate (Dako P0448, Dako, Ely, UK) diluted at 1:2,000 in 0.5% OVA-BST was added. After incubating for one hour and washing, HRP substrate was added (100 μ L per well) and incubated for 10 minutes. The reaction was stopped with 100 μ L sulphuric acid and

optical density (OD) read at 450 nm. The net OD reading for each sample was calculated by subtracting the OD in the control well from that in the matching test well to exclude non-specific background binding.

In order to be able to compare OD values obtained from different plates on different days, we prepared an in-house standard positive control sample that was loaded in serial dilutions (range 1:15 to 1:120) on every plate. This in-house standard was prepared by pooling serum samples from several patients who had been found to have high serum NN levels in this assay. The mean net OD from duplicate test samples was converted to absorbance units (AU) by comparison to the standard curve of OD for the serial dilutions of the positive control sample on each Plate. A total of 100 AU was defined as the OD given by a 1:30 dilution of the positive control sample. The OD for this 1:30 dilution was reproducibly high, ranging between 1.03 and 1.37.

The NN assay was reproducible with an intra and inter-plate coefficient of variation of <10%.

Capture ELISA to detect nitrated albumin

This ELISA protocol was identical to the NN capture ELISA with the following exceptions. The blocking agent was 0.5% agarose in PBS and the anti-histone H3 antibody used was replaced with a rabbit polyclonal anti-human albumin antibody (Abcam 2406).

Direct ELISA to detect anti-nucleosome antibodies (IgG)

All steps were carried out at 37°C. The test side of the plate was coated with 50 µL per well of nucleosome antigen (Arotec ATN02-02, Binding Site Ltd, Birmingham, UK) diluted 1:500 in 20 mM Tris/HCL buffer (pH 8.0) containing 0.15 M of NaCl; the control side was coated with buffer alone. Incubation for two hours, then blocking with 1% bovine serum albumin in PBST (BSA-PBST) for one hour were followed by sample loading in duplicate into test and control wells at 1:50 dilution in BSA-PBST. As the positive control, a pooled serum sample was serially diluted (range 1:15 to 1:120) and loaded in the same way. After a 30-minute incubation, goat polyclonal anti-human IgG HRP conjugate (A6029, Sigma, Dorset, UK) diluted at 1:1,000 in BSA-PBST was added. After incubating for 30 minutes, HRP substrate was added and subsequent steps were as for the NN ELISA.

Statistical methods

The three outcomes in the analyses were serum NN levels, NA levels and anti-nuc levels. All outcomes were found to have a highly positively skewed distribution, which could not be transformed to a more normally distributed scale. Thus, the outcomes were assumed to follow a negative binomial distribution. Due to the longitudinal nature of the cohort, multiple samples for each patient were considered. To allow for the non-independence of the data, multilevel

statistical methods were used for analysis. Two level models were used with individual measurements clustered within patients. The analyses, performed using multilevel negative binomial regression, were performed in two stages. First, the separate effect of each factor upon the outcome was examined in a series of univariable analyses. Subsequently, the joint effect of factors was examined in a multivariable analysis. A backward selection procedure was employed to retain only the statistically significant variables. Variance inflation factors were used to assess collinearity between variables and, as a result, some variables that were collinear with other variables were excluded from the multivariable stage of the analysis.

Additional analyses grouped patients as either NN positive or negative depending on their nitrated nucleosome values. Variables measured at the patient level were compared between groups using either Fisher's exact test for the categorical variables or the unpaired t-test for continuous variables.

Sample level variables were analyzed using multilevel regression methods to allow for the repeat measurements from each patient. Multilevel logistic regression was used to compare binary variables between groups, while multilevel linear regression was used for continuous variables. Continuous variables found to have a positively skewed distribution were given a log transformation before the analysis.

Results

Characteristics of subjects

The mean age of the patients with SLE at the time of the earliest sample assayed was 36 years (SD 13.0) and 81% were female. A total of 23 were Caucasian, 18 Afro-Caribbean and 8 other ethnicities. For the healthy controls, the mean age was 31.6 years (SD 6.0) and 62% were female. A total of 27 were Caucasian, 1 Afro-Caribbean and 6 other ethnicities.

Of the 49 patients with SLE, 21 were anti-Ro positive, 6 anti-La positive, 15 anti-RNP positive and 11 anti-Sm positive. During the follow-up period, 29 patients had at least one elevated anti-dsDNA, 32 had at least one low C3 and 46 suffered at least one flare (BILAG A or B in at least one system). Flares in all eight systems of the classic BILAG index were represented in the cohort.

Serum NN levels are higher in patients with SLE than in healthy controls or patients with other autoimmune diseases

Figure 1 shows that mean levels of serum NN in patients with SLE were significantly higher in patients with SLE than in healthy controls or patients with other autoimmune diseases - rheumatoid arthritis, myositis or Sjogren's syndrome ($P = 0.01$ by one-way ANOVA/Kruskal Wallis test). We do not have formal disease activity measurements for the patients with these other diseases but in most cases, samples

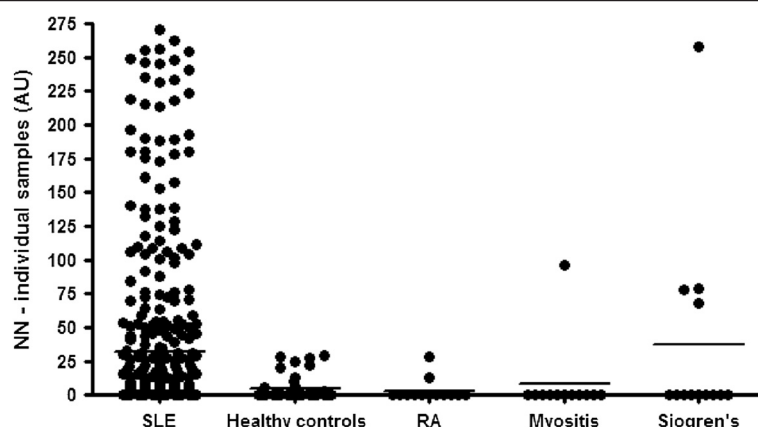


Figure 1 NN levels in patients with SLE compared to healthy and autoimmune disease controls. This figure shows the results obtained in the NN ELISA on testing serum samples from patients with SLE in comparison with healthy controls and patients with other autoimmune rheumatic diseases (myositis, rheumatoid arthritis and Sjogren's syndrome). NN levels were higher in the SLE group than the other groups ($P = 0.01$ by one way ANOVA/Kruskal Wallis test). NN, nitrated nucleosomes; SLE, systemic lupus erythematosus.

were taken when patients were symptomatic. Since NN levels were very low in 31/38 of these patients, we do not believe that active rheumatoid arthritis, myositis or Sjogren's syndrome is associated with raised NN levels.

Serum NN are present in 63% of patients with SLE and the levels of NN in these patients vary over time

Of the 49 patients with SLE tested, 18 never had detectable serum NN, whereas the other 31 (63%) had serum NN levels that varied significantly over time (mean 32 AU, SD 62.2, range 0 to 270). The characteristics of the 18 NN-negative patients and the other 31 patients are compared in Table 1. All patients who never had NN were anti-Sm negative, whereas 35% of the others were anti-Sm positive ($P = 0.004$). This clear dichotomy did not apply to any of the other antibody specificities that were tested, though the mean anti-dsDNA level in NN-positive patients was almost twice that seen in NN-negative patients. A total of 63.2% of NN-positive samples, but only 25.2% of NN-negative samples, came from patients who were taking immunosuppressants ($P = 0.001$).

Figure 2 shows the variation of serum NN levels over time in five patients with SLE (patients SLE 9, 15, 18, 41 and 42) compared to variations in anti-dsDNA level (Figures 2A-E) and global BILAG score (Figures 2F-J). None of these patients showed close relationships between anti-dsDNA and NN over time. In some cases, such as SLE 9, 15 18 and 42, NN levels follow disease activity more closely than anti-dsDNA. This is especially striking in cases SLE 15 and 42, where anti-dsDNA is always low. However, there are other cases where the NN level is persistently low and anti-dsDNA follows activity more closely (patient SLE 41) and it is important to remember that approximately a third of patients never had raised NN levels.

Clinical, demographic and serological variables associated with serum NN and NA levels in patients with SLE

Our hypothesis was that the levels of NN in patients with SLE would be affected both by the overall level of nitration and by the level of serum nucleosomes present as a target for nitration. If this were true, there should be detectable differences between the variables influencing serum NN levels and those influencing NA levels. These differences clearly exist, as shown in Table 2, which includes results from univariable analysis of 24 factors.

NN levels were twice as high in men as in women. NA levels were five times as high in men as in women. The effects of ethnicity on NA are the opposite of the effects on NN. Caucasians had the lowest NA levels but highest NN levels of any ethnic group. For both NN and NA there was a complex non-linear relationship with both disease duration and age at diagnosis. NN levels were lowest in very young and very old subjects with a peak NN level at age 30 and at disease duration of eight years.

Anti-Sm positivity was strongly associated with both elevated NN and elevated NA.

We did not detect any association with the commonly used serological markers of active lupus (elevated anti-dsDNA and low C3) or with persistent overall disease activity for either NN or NA. Considering the individual systems of the body, we found that vasculitis flares (that is, A or B score in the vasculitis domain of BILAG) were associated with significantly raised serum NN and NA. NN but not NA was raised in cardiorespiratory flares. Renal flares were associated with a significant decrease in NA but not in NN. There were no statistically significant differences between flare (A/B) and non-flare (C/D/E) samples for other systems but for neuropsychiatric flares the NN levels in C/D/E samples were approximately 50% of those in A/B samples. This difference did not reach statistical significance due to wide

Table 1 Comparison of patients with SLE who never had serum NN with the rest of the group

	NN-negative (n patients = 18) (n samples = 144)	NN-positive (n patients = 31) (n samples = 253)	P-value
Mean age at first sample (SD) ^(*)	37.7 (13.1)	34.9 (12.8)	0.47
% Female (n) ^(*)	77.8 (14)	83.9 (26)	0.71
Ethnicity ^(*)			0.41
% AC (n)	27.8 (5)	41.9 (13)	
% C (n)	61.1 (11)	38.7 (12)	
% Other (n)	11.1 (2)	19.3 (6)	
Median anti-dsDNA IU/ml (IQR) ^(**) (†)	29 (13, 97)	82 (17, 171)	0.18
Mean C3 g/l (SD) ^(**)	1.0 (0.2)	0.9 (0.3)	0.10
Median Global BILAG score (IQR) ^(**) (†)	5 (2, 8)	5 (3, 10)	0.22
% anti-Sm positive (n) ^(*)	0.0 (0)	35.5 (11)	0.004
% anti-RNP positive (n) ^(*)	27.8 (5)	32.3 (10)	1.00
% anti-Ro-positive (n) ^(*)	38.9 (7)	45.2 (14)	0.77
% anti-La positive (n) ^(*)	16.7 (3)	9.7 (3)	0.66
% on hydroxychloroquine (n) ^(**)	42.1 (61)	44.7 (113)	0.66
% on immunosuppressants (n) ^(**)	25.7 (37)	63.2 (160)	0.001
% on ≥5 mg/day corticosteroids (n) ^(**)	68.1 (98)	78.3 (198)	0.31

^(*)Analysis performed at the patient level.

^(**)Analysis performed at the sample level.

^(†)Statistical comparison performed on log scale.

Footnote - Global BILAG score is calculated from the formula A = 9, B = 3, C = 1, D = 0, E = 0 as described in previous papers [12]. Bold type indicates statistically significant P-values ($P < 0.05$). BILAG, British Isles Lupus Assessment Group; NN, nitrated nucleosomes; RNP, ribonucleoprotein; SLE, systemic lupus erythematosus.

confidence intervals. There was no effect of neuropsychiatric flares on NA levels.

Treatment with hydroxychloroquine was associated with elevated NN and NA levels. Immunosuppressants (including mycophenolate, azathioprine, methotrexate and cyclophosphamide) were only associated with elevated NN, whereas high dose corticosteroid treatment was associated with neither NN nor NA.

Tables 3 and 4 show the results of multivariable analysis of associations with NN and NA, respectively. Disease duration, ethnicity, negative anti-La, albumin and low renal disease activity are all associated with NA but not NN, whereas hydroxychloroquine and high vasculitis disease activity are associated with NN but not NA. Age at diagnosis, anti-Sm antibody positivity and treatment with immunosuppressants all show similar associations with both NN and NA levels. In particular, anti-Sm positivity shows a very strong independent association with both NN and NA ($P < 0.001$ for both).

There is no association between serum levels of NN and anti-nuc in multivariable analysis and different variables are associated with these outcomes

Both univariable and multivariable analyses of the factors associated with serum anti-nuc levels were performed. Only the results of multivariable analysis are presented

here (in Table 5), as use of anti-nuc as a biomarker in SLE has been studied extensively by other authors [13-15] and is not the subject of our paper.

Samples obtained from men had higher anti-nuc levels than samples from women. Both increasing age at diagnosis and increasing disease duration were associated with significant reductions in anti-nuc levels. Increasing anti-nuc levels were associated with elevated anti-dsDNA as noted by previous authors [14,15] and with low C3. In contrast to the results obtained with NN and NA, there was no relationship between anti-nuc and anti-Sm status. There were no associations between treatment with hydroxychloroquine or immunosuppressants and levels of anti-nuc.

Most importantly from the point of view of the present study, there was no significant association between NN and anti-nuc levels on multivariable analysis suggesting that nitration probably does not affect the immunogenicity of nucleosomes.

Discussion

In this paper we describe a novel capture ELISA that measures serum levels of NN. Mean NN levels are significantly higher in patients with SLE than in healthy controls or in patients with other autoimmune rheumatic diseases but there is a subset of patients with SLE who never test positive for NN. These persistently NN-negative patients

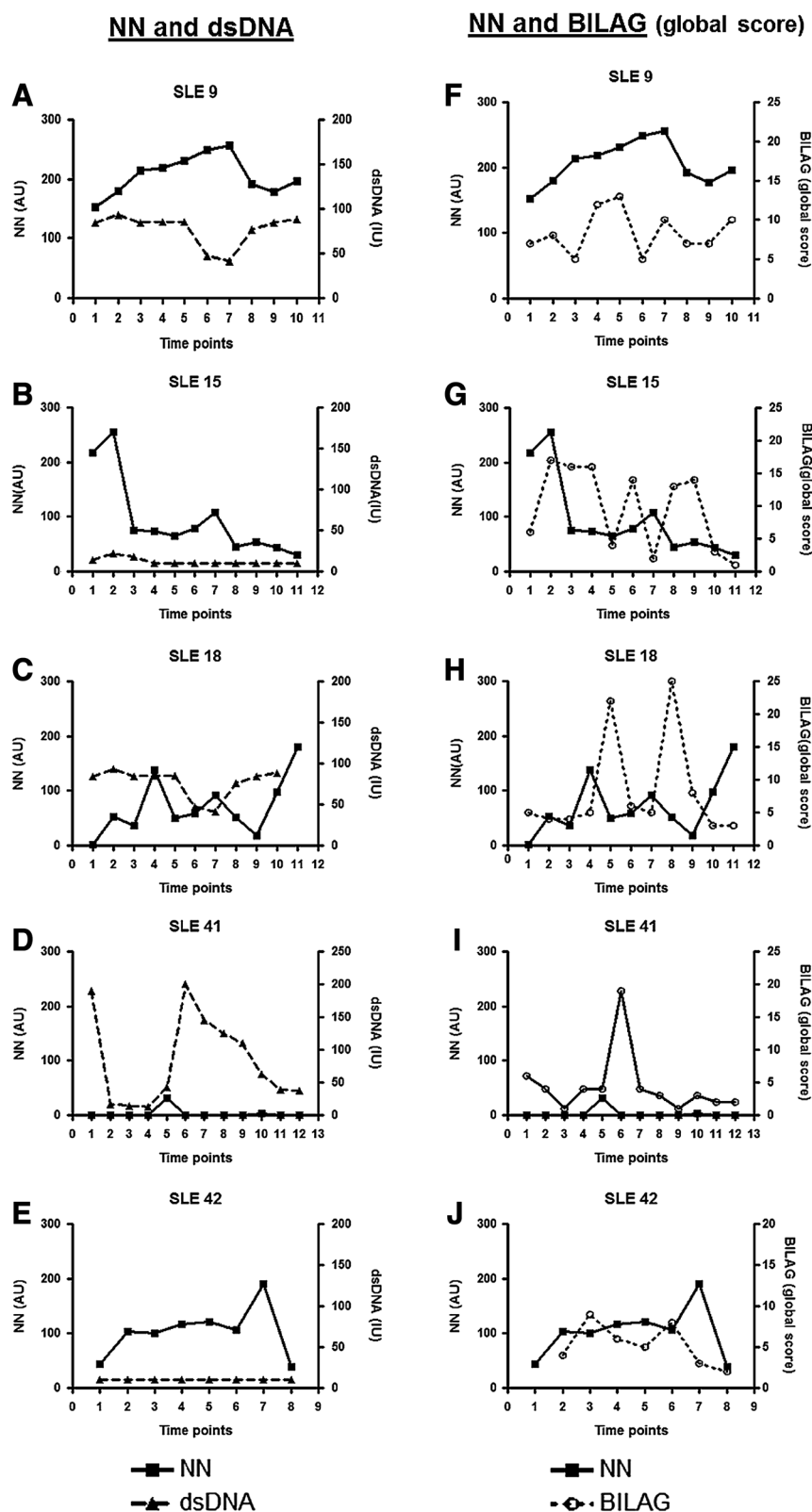


Figure 2 Variation of NN and anti-dsDNA levels and global BILAG scores over time. This figure shows the variation of serum NN levels over time in five patients compared to anti-dsDNA levels (A-E) and global BILAG scores (F-J). anti-dsDNA, anti-double stranded DNA antibodies; BILAG, British Isles Lupus Assessment Group; NN, nitrated nucleosomes.

Table 2 Univariable analysis of factors associated with serum NN and NA levels in patients with SLE

Variable	Category/term	Ratio for NN (95% CI)	P-value for NN	Ratio for NA (95% CI)	P-value for NA
Gender *	Female (n = 336)	1		1	
	Male (n = 61)	2.01 (1.06, 3.81)	0.03	5.00 (2.50,9.98)	<0.001
Disease duration [†]	Ratio given per 5-year increase	0.43 (0.23, 0.79)	0.005	0.32 (0.20,0.51)	<0.001
Age (at diagnosis) [†]	Ratio given per 10-year increase	0.10 (0.02, 0.46)	<0.001	0.04 (0.01,0.16)	0.002
Ethnicity*	Caucasian (n = 182)	1		1	
	Afro-Caribbean (n = 146)	0.75 (0.47, 1.22)		2.07 (1.26,3.40)	
	Other (n = 69)	0.33 (0.17, 0.63)	0.003	3.75 (1.86,7.56)	<0.001
Any ENA**	No (n = 165)	1		1	
	Yes (n = 232)	3.43 (2.19, 5.37)	<0.001	3.19 (1.93,5.27)	<0.001
Anti-Ro**	No (n = 226)	1		1	
	Yes (n = 171)	1.70 (1.12, 2.59)	0.01	1.81 (1.14,2.89)	0.01
Anti-La**	No (n = 345)	1		1	
	Yes (n = 52)	0.79 (0.41, 1.50)	0.47	0.46 (0.20,1.06)	0.07
Anti-Sm**	No (n = 298)	1		1	
	Yes (n = 99)	5.63 (3.66, 8.67)	<0.001	6.25 (3.84,10.2)	<0.001
Anti-RNP**	No (n = 288)	1		1	
	Yes (n = 109)	1.08 (0.69, 1.70)	0.74	1.72 (1.06,2.79)	0.03
Anti-dsDNA level	<50 IU/ml (n = 177)	1		1	
	≥50 IU/ml (n = 170)	0.76 (0.26, 1.13)	0.17	1.32 (0.66,2.65)	0.43
C3 level	<0.9 g/l (n = 139)	1		1	
	≥0.9 g/l (n = 208)	0.83 (0.57, 1.20)	0.32	1.26 (0.86,1.87)	0.24
Disease activity in general system§	A, B (n = 31)	1		1	
	C, D, E (n = 344)	0.96 (0.60, 1.54)	0.87	1.19 (0.71,2.00)	0.52
Disease activity in mucocutaneous system	A, B (n = 41)	1		1	
	C, D, E (n = 334)	0.99 (0.63, 1.56)	0.95	1.32 (0.76,2.29)	0.33
Disease activity in neuropsychiatric system	A, B (n = 18)	1		1	
	C, D, E (n = 357)	0.56 (0.13, 2.38)	0.44	1.04 (0.50,2.19)	0.91
Disease activity in musculoskeletal system	A, B (n = 47)	1		1	
	C, D, E (n = 328)	1.20 (0.47, 3.04)	0.71	0.68 (0.43,1.05)	0.08
Disease activity in cardiorespiratory system	A, B (n = 13)	1		1	
	C, D, E (n = 362)	0.43 (0.24, 0.78)	0.006	1.40 (0.22,8.70)	0.72
Disease activity in vascular system	A, B (n = 12)	1		1	
	C, D, E (n = 363)	0.33 (0.17, 0.63)	0.001	0.39 (0.19,0.82)	0.01
Disease activity in renal system	A, B (n = 41)	1		1	
	C, D, E (n = 329)	1.09 (0.40, 2.97)	0.86	4.28 (2.13,8.58)	<0.001
Disease activity in hematological system	A, B (n = 95)	1		1	
	C, D, E (n = 280)	1.21 (0.84, 1.74)	0.30	1.44 (0.95,2.17)	0.08
Overall disease activity over last four assessments	Persistently low (n = 166)	1		1	
	Persistently mod/high (n = 209)	0.91 (0.66, 1.25)	0.57	0.66 (0.47,0.92)	0.01
Hydroxychloroquine	No (n = 223)	1		1	
	Yes (n = 174)	3.52 (2.40, 5.17)	<0.001	3.21 (2.08,4.95)	<0.001
Immunosuppression	No (n = 200)	1		1	
	Yes (n = 197)	1.66 (1.17, 2.34)	0.004	1.33 (0.89,1.98)	0.16

Table 2 Univariable analysis of factors associated with serum NN and NA levels in patients with SLE (Continued)

Oral corticosteroids	≤7.5 mg/day (n = 101)	1		1	
	>7.5 mg/day (n = 296)	0.81 (0.59, 1.13)	0.22	1.00 (0.69,1.44)	0.99
Albumin	Ratio given per 5 g/l increase	1.38 (1.20,1.59)	<0.001	1.42 (1.22,1.65)	<0.001

*For gender, n values refer to the numbers of samples taken from female and male subjects, rather than the numbers of females and males in the cohort of patients. A similar stipulation applies to ethnicity where n values refer to the number of samples taken from patients of each ethnic group.

†For the complex associations with disease duration and age, we carried out analysis of both linear and squared terms. The figures in the table refer to linear terms. The ratios for squared terms for NN are 0.66 (95% CI: 0.51, 0.85) for disease duration and 1.56 (95% CI: 1.20, 2.03) for age. The ratios for squared terms for NA are 0.59 (95% CI: 0.46, 0.75) for disease duration and 1.72 (95% CI: 1.32, 2.24) for age.

**For ENA, anti-Ro, anti-La and anti-Sm we did not have results from the date of every sample but it is assumed that positivity and negativity for these antigens generally remain stable.

§“Disease activity in general system” refers to the BILAG score (A, B, C, D or E) in the General Category of the BILAG index on the day when each sample was taken. The same principle applies to the remaining organ systems listed in the table, which are the eight different categories recorded in BILAG. Bold type indicates statistically significant *P*-values (*P* <0.05).

comprise about one-third of the total population and have lower disease activity and anti-dsDNA antibody levels and less use of immunosuppressants than the other two-thirds. NN-negative patients are all anti-Sm antibody negative.

Currently, available serum biomarkers for monitoring patients with SLE are antibodies (particularly anti-dsDNA) or markers of immune activation, such as depleted complement levels. Potential new serum biomarkers include pro-inflammatory chemokines [16]. The ELISA described in our current paper is novel in that, rather than an antibody or cytokine, it measures levels of an antigen, which has been chemically modified *in vivo*. The critical importance of nucleosomes in the pathogenesis of lupus nephritis has been established by a number of authors. Berden and colleagues showed that nucleosome/anti-nucleosome complexes can cause nephritis in murine models of SLE [9,17]. The elegant electron microscopy studies of Rekvig's group demonstrated that deposited IgG co-localizes with electron-dense chromatin structures in renal biopsies from human and murine lupus nephritis [18,19]. More recently, Kanapathipillai *et al.* showed a direct stimulatory effect of nucleosomes alone (not requiring complexed antibodies) on expression of chemokines by

mesangial cells from NZB/W F1 mice [20]. There is also evidence that chemical modification of nucleosomes, specifically hyperacetylation, can alter their biological effects in SLE. Dieker *et al.* demonstrated that samples from 26 of 35 patients with SLE bound more strongly to a triacetylated 22-amino acid peptide from histone H4 than to the non-acetylated version of the same peptide [21]. Subcutaneous administration of the triacetylated, but not the non-acetylated, peptide accelerated development of proteinuria and increased mortality in MRL/*lpr* mice [21] and this effect was not mediated via an increase in anti-nucleosome antibody levels. In the same paper, this group showed that hyperacetylated nucleosomes stimulated expression of co-stimulatory molecules and pro-inflammatory cytokines by dendritic cells (DC) from MRL/*lpr* mice [21]. However, they

Table 4 Multivariable analysis of factors associated with serum NA levels in patients with SLE

Variable	Category/term	Ratio (95% CI)	<i>P</i> -value
Disease duration	Ratio given per five-year increase	2.24 (1.55, 3.26)	<0.001
Age (at diagnosis) *	Age	0.01 (0.00, 0.04)	<0.001
	Age ²	2.58 (1.86, 3.56)	
Ethnicity	Caucasian	1	
	Afro-Caribbean	2.99 (1.35,6.65)	
	Other	14.9 (5.82, 38.0)	<0.001
Anti-La	No	1	
	Yes	0.16 (0.06, 0.49)	<0.001
Anti-Sm	No	1	
	Yes	28.2 (14.4, 55.3)	<0.001
Disease activity in renal system	A, B	1	
	C, D, E	3.78 (2.07,6.90)	<0.001
Immunosuppression	No	1	
	Yes	1.45 (1.02, 2.05)	0.04
Albumin	Ratio per 5 g/l increase	1.12 (1.01,1.24)	0.03

Table 3 Multivariable analysis of factors associated with serum NN levels in patients with SLE

Variable	Category/term	Ratio (95% CI)	<i>P</i> -value
Age (at diagnosis) *	Age	0.02 (0.003, 0.11)	<0.001
	Age	2.12 (1.64, 2.76)	
Anti-Sm	No	1	
	Yes	6.31 (3.39, 11.7)	<0.001
Disease activity in vascular system	A, B	1	
	C, D, E	0.40 (0.24, 0.69)	0.001
Hydroxychloroquine	No	1	
	Yes	1.96 (1.09, 3.53)	0.02
Immunosuppression	No	1	
	Yes	2.96 (1.97, 4.46)	<0.001

*Ratio given for 10-year increase. NN, nitrated nucleosomes; SLE, systemic lupus erythematosus.

*Ratio given for a 10-year increase. NA, nitrated albumin; SLE, systemic lupus erythematosus.

Table 5 Multivariable analysis of factors associated with serum anti-nuc levels in patients with SLE

Variable	Category/term	Ratio (95% CI)	P-value
Gender	Female	1	
	Male	2.83 (1.72, 4.69)	<0.001
Disease duration	Ratio given per 5-year increase	0.87 (0.78, 0.98)	0.02
Age (at diagnosis)	Ratio given per 10-year increase	0.59 (0.49, 0.72)	<0.001
RNP	No	1	
	Yes	0.47 (0.32, 0.70)	<0.001
dsDNA	Ratio given per 10-fold increase (1 unit on log scale)	1.79 (1.40, 2.28)	<0.001
C3	Ratio given per 0.1 g/l increase	0.29 (0.15, 0.56)	<0.001
NN level	Ratio given per 10-fold increase (1 unit on log scale)	0.88 (0.72,1.07)	0.20

Anti-nuc, Anti-nucleosome antibody; dsDNA, double stranded DNA; RNP, ribonucleoprotein; SLE, systemic lupus erythematosus.

did not measure levels of hyperacetylated nucleosomes in the serum of humans or mice. Our current paper is the first to look specifically at nitration of nucleosomes and the first to measure serum levels of modified nucleosomes in patients with SLE. We found that elevated NN level is itself a specific marker for SLE and is also strongly associated with another SLE-specific biomarker in anti-Sm. Though these are promising findings, limitations of our work include the absence of strong association with overall disease activity and expression of results in terms of binding of our in-house control serum sample. If NN is to be a useful biomarker in patients with SLE, a more generally applicable standard must be found. In a recently published paper, Pratesi *et al.* [22] showed that sera from patients with rheumatoid arthritis, but not from healthy controls or patients with other autoimmune rheumatic diseases, contained antibodies to deiminated histone H4. They postulated that the deiminated H4 autoantigens could derive from neutrophil extracellular traps in these patients. A modification of our NN assay, using anti-citrulline rather than anti-nitrotyrosine as the capture antibody, could potentially be used to detect deiminated histones in the serum of patients with rheumatoid arthritis.

There was a strong association between anti-Sm positivity and nitration in the multivariable analysis of factors associated with NN and NA levels. To our knowledge this association between nitrative stress and anti-Sm positivity has not been described before. The Sm antigen is a complex of ribonucleoproteins in the spliceosome [23]. Anti-Sm antibodies were first recognized in 1966 [24], and are highly specific to patients with SLE. Anti-Sm antibodies have been found in lupus nephritis renal biopsies [25]. Some retrospective studies reported associations between anti-Sm positivity and psychosis [26] or nephritis [27], while others found no association between anti-Sm antibodies and any clinical outcome [28]. A large Chinese study compared 469 anti-Sm antibody positive and 1,115 anti-Sm antibody negative patients with SLE [29]. The anti-Sm positive patients had higher disease activity,

higher anti-dsDNA antibody and lower C3 levels than anti-Sm negative patients. Vasculitis occurred more often in anti-Sm positive than in anti-Sm negative patients (13.7% vs 7.4%, $P < 0.05$). Our current results show strong associations among NN levels, anti-Sm and vasculitis.

Increased nitration may promote development of anti-Sm antibodies. Experiments on mice transgenic for the rearranged heavy chain variable region genes of a monoclonal anti-Sm antibody [30] showed that immature DC carrying surface Sm antigen interacted directly with B cells leading to the production of anti-Sm antibody. These DC obtained Sm antigen partly from apoptotic cell debris, which also contains nucleosomes complexed to the High Mobility Group Box 1 (HMGB1) protein [31]. HMGB1 can activate DC [32] and also promotes NO production by phagocytes. Thus nucleosome/HMGB1 complexes could stimulate the DC-B cell interaction that leads to anti-Sm antibody production as well as driving NO production. It is not known whether nitration of HMGB1 itself alters its pro-inflammatory properties. However, a recent study [33] has shown that altering the redox state of HMGB1 through the oxidation of free thiol cysteines to form disulfide bonds makes it more pro-inflammatory. If nitration of nucleosome/HMGB1 complexes likewise promotes their pro-inflammatory properties this would create a potential positive feedback loop, and this is a mechanism that warrants further exploration. It may also be interesting to look at nitration of the Sm antigen.

NA and NN levels were associated with use of immunosuppressants. This association is unlikely to arise simply from the fact that immunosuppressants are used more commonly in highly active disease, because persistent clinical disease activity, anti-dsDNA antibody and C3 levels were not associated with either NA or NN levels. In future studies it may also be interesting to look at correlations with erythrocyte sedimentation rate and/or C-reactive protein but we do not have data on those variables for this study. We collected data on anti-dsDNA and C3 because those biomarkers are more closely associated with flares in

SLE. The drugs themselves could promote nitration, but we know of no evidence supporting this. Cyclosporin can promote tyrosine nitration in endothelial cells [34], but only two samples in this study were from patients taking cyclosporin and both were negative for NN.

A large inception cohort study has shown that 40% of 1,206 patients with SLE suffered from neuropsychiatric symptoms, which were associated with reduced quality of life but that in the majority of cases these were not due to active inflammation and did not require immunosuppression [35]. It is important, however, to identify the subpopulation of patients in whom these symptoms are due to active cerebral SLE. With appropriate treatment, the symptoms are significantly more likely to resolve in those patients compared to patients in whom the symptoms are not due to active SLE [35]. Current imaging techniques and blood tests do not accurately distinguish patients with active neuropsychiatric SLE from those who have neuropsychiatric symptoms not caused by SLE. Our results suggesting that elevated NN levels could potentially be a marker for neuropsychiatric flares are based on results from 11 different patients taken over a 12-year period. Over 70% of the neuropsychiatric manifestations in these patients were headaches and seizures. It is important to extend these results by testing larger numbers of samples from patients with a wider range of neuropsychiatric manifestations.

Vasculitis is a relatively uncommon manifestation of SLE [29]. In multivariable analysis we found a statistically significant association of high NN (but not NA) with vasculitis based on results from nine different patients over an 11-year period. Review of the medical records showed that all these patients had cutaneous vasculitis at the time of their flares. It is possible that raised NN levels in patients without visible vasculitis may be a marker of subclinical vascular activation. It will, therefore, be interesting to see whether NN levels are associated with objective measures of atherosclerosis, such as carotid ultrasound [2]. This potential association is underlined by the fact that peroxynitrite, a powerful nitrating and oxidizing agent, is generated in atherosclerotic plaques. If such an association exists, then NN levels may be relevant to assessment of CVD risk in patients with SLE.

Conclusion

By developing a novel assay to measure serum NN levels we have demonstrated that these levels are raised in patients with SLE compared to healthy controls and patients with other autoimmune rheumatic diseases. NN-positivity is strongly linked to anti-Sm antibody positivity and may be a marker for neuropsychiatric flares and vasculitis in patients with SLE. Further studies in larger numbers of patients with these manifestations are required.

Abbreviations

Anti-dsDNA: Anti-double stranded DNA antibodies; Anti-nuc: Anti-nucleosome antibody; AU: Absorbance units; BILAG: British Isles Lupus Assessment Group index (for measuring disease activity in SLE); BSA-PBST: Bovine serum albumin in PBST; CVD: Cardiovascular disease; DC: Dendritic cells; ELISA: Enzyme-linked immunosorbent assay; HMGB1: High Mobility Group Box 1 protein; HRP: horse radish peroxidase; NA: Nitrated albumin; NN: Nitrated nucleosomes; NO: Nitric oxide; OD: Optical density; OVA-BST: Ovalbumin in PBST; PBS: Phosphate-buffered saline; PBST: PBS-0.1% Tween; SLE: Systemic lupus erythematosus; UCLH: University College London Hospital.

Competing interests

The authors have no competing interests.

Authors' contributions

YI and AR conceived the idea of the project. YI, CP and KFA developed and optimized the novel nitrated nucleosome assay. SC carried out the experimental work. PB and SC carried out statistical analysis. IG, DI, YI, AR, SC and DL took part in the design of the study and analysis of data. SC and AR wrote the final paper. All authors revised the paper and agreed to the final version.

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RESEARCH ARTICLE

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IgG anti-apolipoprotein A-1 antibodies in patients with systemic lupus erythematosus are associated with disease activity and corticosteroid therapy: an observational study

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Abstract

Introduction: IgG anti-apolipoprotein A-1 (IgG anti-apoA-1) antibodies are present in patients with systemic lupus erythematosus (SLE) and may link inflammatory disease activity and the increased risk of developing atherosclerosis and cardiovascular disease (CVD) in these patients. We carried out a rigorous analysis of the associations between IgG anti-apoA-1 levels and disease activity, drug therapy, serology, damage, mortality and CVD events in a large British SLE cohort.

Methods: Serum IgG anti-apoA-1 levels were measured in 100 healthy controls to define a cut-off for positivity. In 499 patients with SLE we obtained the earliest stored serum sample from their disease course and measured IgG anti-apoA-1 level. We then examined associations between IgG anti-apoA-1 positivity in early disease and the development of damage, CVD or death over a mean follow-up period of 12.1 years in these patients. In a separate study, we measured IgG anti-apoA-1 levels in 397 samples taken longitudinally from 49 patients with SLE over a mean period of 89 months of fluctuating disease activity and carried out multi-variable analysis to examine the demographic, serological, disease activity and treatment factors associated with IgG anti-apoA-1 level over time.

Results: In the longitudinal study, IgG anti-apoA-1 levels were significantly higher in patients with persistently active disease, those on high dose corticosteroid and those not taking hydroxychloroquine. Of the 499 subjects who had early disease IgG anti-apoA-1 levels measured, 135 (27%) were positive. However, we found no convincing associations between early IgG anti-apoA-1 positivity and development of damage, mortality or CVD.

Conclusions: IgG anti-apoA-1 developed early in a quarter of our patients with SLE, but this had no major impact on subsequent clinical outcomes. However, levels of IgG anti-apoA-1 vary over time and are associated with disease activity, treatment with high dose corticosteroid and not taking hydroxychloroquine.

Introduction

Antibodies to apolipoprotein A-1 (anti-apoA-1) have been reported in the serum of patients with systemic lupus erythematosus (SLE) [1-4]. Several groups found independently that levels were elevated in patients with active SLE compared with inactive SLE [1-4], although none carried out multivariable analysis to exclude the

effect of confounding factors. Apolipoprotein A-1 is the main constituent of high-density lipoprotein, which plays a major role in preventing atherosclerosis [5,6]. It has been suggested that anti-apoA-1 could interfere with this protective effect and thus promote atherosclerosis [2,4]. IgG anti-apoA-1 have also been associated with markers of increased plaque vulnerability such as intra-plaque macrophage, neutrophil and matrix metalloproteinase content [7] and are associated with increased levels of the surrogate plaque vulnerability markers interleukin-6, tumour necrosis factor alpha and matrix metalloproteinase-9 in patients

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with myocardial infarction [8]. Elevated IgG anti-apoA-1 levels are found in patients with acute coronary syndromes [9], are a risk factor for major cardiovascular events in the 12 months after myocardial infarction [10] and are associated with significantly increased risk of major cardiovascular events in patients with rheumatoid arthritis (RA) [11]. Anti-apoA-1 antibodies have thus attracted particular interest as a possible mediator between inflammation and the recognised increased risk of developing cardiovascular disease (CVD) in patients with SLE.

In a large multinational study of 9,547 patients with SLE there were 1,255 deaths, of which 313 were due to CVD [12]. Patients with SLE have fivefold to 10-fold greater risk of developing CVD than age-matched controls [13], rising to 50-fold in women between the ages of 35 and 44 [14]. Subclinical vascular disease is more common in patients with SLE than in age-matched and sex-matched controls [15,16]. Patients with SLE who suffer CVD events do so at a relatively young age – a mean of 49 years for women [13]. Standard methods based on the Framingham equations underestimate CVD risk in patients with SLE [17,18]. Alternative stratification tools to identify atherosclerosis – for example, vascular ultrasound – may be useful [19,20]. If IgG anti-apoA-1 have a true predictive value for CVD this could also be helpful. Previously, we did not find high IgG anti-apoA-1 levels in 24 patients with SLE who suffered CVD events [2]. Radwan and colleagues found no relationship between IgG anti-apoA-1 and carotid intima-media thickness in 80 Egyptian patients with SLE but, unlike most other SLE groups studied, none of their patients had carotid plaques [4].

Previous studies of IgG anti-apoA-1 in patients with SLE were limited by having small numbers of patients, lack of multivariable analysis and short-term follow-up after the samples were taken. Here, we address these issues to answer the following questions: which demographic, serological, clinical and treatment factors are significantly associated with changing IgG anti-apoA-1 levels over time in patients with SLE? What is the prevalence of positivity for IgG anti-apoA-1 early in the disease course of SLE? Does positivity for IgG anti-apoA-1 early in the disease course predict subsequent damage, cardiovascular disease and/or mortality?

Methods

Ethical approval for all parts of the study was granted by the joint University College London/University College London Hospitals Research Ethics Committee (Reference 06/Q0505/79) and subjects gave informed consent for use of their stored serum samples.

Healthy control subjects

We tested samples from 100 healthy control subjects originally obtained as part of the Health Survey for England

2006 [21]. The samples were provided to us by the Health and Social Care Information Centre together with anonymised data on age, and the gender and ethnicity of the subjects and absence of long-term illness or previous CVD were confirmed. Their median age was 43 years (range 20 to 69), 49% were female and the ethnic distribution was 83% Caucasian, 8% Afro-Caribbean and 9% South Asian.

Patients with systemic lupus erythematosus

The University College London Hospitals SLE clinic has been running since 1979 and we have followed over 600 patients with SLE since then, all fulfilling the revised American College of Rheumatology classification criteria [22]. From this population of patients we selected two different groups who were studied to answer different questions.

Early disease group

For 499 patients we were able to obtain serum samples taken within 1 year of diagnosis and stored at -80°C . The earliest of these samples was obtained in 1978 and the most recent in 2011. We tested all of these samples for IgG anti-apoA-1 antibodies – using the enzyme-linked immunosorbent assay (ELISA) described below – to find out what proportion of patients with SLE have IgG anti-apoA-1 early in the disease course. These 499 patients with SLE had been under continuous follow-up for between 1 and 34 years (mean 12.1 years). We investigated whether positivity for IgG anti-apoA-1 within the first year of the disease was associated with the following outcomes: death from any cause; death before the age of 60; CVD – defined as either myocardial infarction with typical enzyme and/or ECG changes, ischaemic stroke confirmed by imaging or non-infarct coronary disease confirmed by angiography; and damage as measured by the Systemic Lupus International Collaborative Clinics Damage Index (SLICC-DI) [23].

Data on death and CVD were available for all 499 patients from review of their clinical records. Data on SLICC-DI scores were only available for 236 patients. These patients had been studied between 2006 and 2008 in a project designed specifically to collect comprehensive data on damage scores from patients who had been followed in the clinic for at least 10 years by then (that is, had been diagnosed between 1979 and 1996) [24]. Only 236 such patients were available and thus only SLICC-DI data from those patients are included in the current report.

For the early disease samples we did not have data on other autoantibodies, or on the disease activity or damage scores at the time of the sample.

Longitudinal group

Longitudinal serum samples ($n = 397$) were selected retrospectively from a group of 49 patients with SLE with a mean of eight samples per patient (standard deviation (SD) 2.16; minimum 3; maximum 14) that had been obtained during a mean period of 89 months (SD 46; minimum 14; maximum 180) in the course of their disease. The patients were selected on the basis that they had varying levels of disease activity over time, including examples of flares in all of the main organs and systems of the body. By coincidence, 47 patients in this group were also members of the early disease group, but the longitudinal serum samples used in this part of the project were different from the early disease samples used in the other part of the project.

For all samples where data were available (94%), we obtained anti-dsDNA and complement C3 levels and disease activity from the date of the sample and from the previous three assessments. Anti-dsDNA and C3 were measured in the routine clinical laboratory at University College London Hospitals using the ELISA (Shield Diagnostics, Dundee, UK) and laser nephelometry respectively. Based on the normal limits for our laboratory, anti-dsDNA level >50 IU/ml was defined as high and C3 level <0.9 g/l as low.

Disease activity was measured using the classic British Isles Lupus Assessment Group (BILAG) index [25]. The more recent BILAG 2004 index was not used because many of the samples had been taken before 2004. Disease activity over the most recent four assessments was characterised as persistently low activity (all systems BILAG C, D or E) or persistently moderate–high activity (A or ≥ 1 B in any BILAG system on at least 2/4 occasions). Over 90% of all samples fell into one of those two categories and the rest were excluded from this part of the analysis. Global BILAG score was calculated using the formula $A = 12$, $B = 5$, $C = 1$, $D = E = 0$ as described previously [26].

Data on ethnicity, gender, drug therapy and the anti-Sm, anti-RNP, anti-Ro and anti-La (all tested by ELISA) status of the patients were obtained from the clinical records of the patients. We did not have data on SLICC-DI score at the time of each sample.

Direct ELISA to detect IgG anti-ApoA-1 antibodies

IgG anti-ApoA-1 antibodies were detected by a modification of the direct ELISA protocol described previously [2,27]. All steps were carried out at 37°C except where specified. A Nunc-Maxisorb 96-well ELISA (Fisher Scientific, Loughborough, UK) plate was divided in half. One side (the test side) was coated with $10\text{ }\mu\text{g/ml}$ apolipoprotein A-1 (A0722; Sigma St Louis, Missouri, USA) in 70% ethanol. The other side (the control side) was coated with 70% ethanol. After incubation for 90 minutes, the plates were washed and blocked with 1% bovine serum albumin diluted in

phosphate-buffered saline for 1 hour. Serum samples at 1:50 dilution in 1% bovine serum albumin–phosphate-buffered saline were tested in duplicate such that each sample was added to two test wells and two control wells. On each plate, a seven-point dilution of the positive control (a pool of six serum samples from patients known to have high serum IgG anti-apoA-1) was performed starting at 1:25 dilution. Following incubation for 1 hour, goat anti-human IgG–alkaline phosphatase conjugate (A3150; Sigma) diluted 1:1,000 in 1% bovine serum albumin–phosphate-buffered saline was added at room temperature for 1 hour followed by alkaline phosphatase substrate. Absorbance at 405 nm was recorded after 60 minutes. The net optical density (OD) reading for each sample was calculated by subtracting the OD in the control well from that in the matching test well to exclude nonspecific background binding. The mean net OD from the duplicate samples was converted to absorbance units (AU) by comparison with the standard curve of OD for the serial dilutions of the positive control sample on each plate. A value of 100 AU was defined as the OD given by a 1:50 dilution of the positive control sample. This assay was reproducible with intraplate and interplate coefficients of variation $<10\%$.

Statistical analysis

In the longitudinal group, assessment of anti-ApoA-1 levels showed a highly positive skewed distribution, which could not be transformed to a normally distributed scale. The outcomes were thus assumed to follow a negative binomial distribution. Owing to the longitudinal nature of this group, multiple samples for each patient were considered. To allow for the non-independence of the data, multilevel statistical methods were used for analysis. Two-level models were used with individual measurements clustered within patients. The analyses, performed using multilevel negative binomial regression, were performed in two stages. Firstly the separate effect of each factor upon the outcome was examined in a series of univariable analyses. Subsequently the joint effect of factors was examined in a multivariable analysis. A backward selection procedure was employed to retain only the statistically significant variables. Variance inflation factors were used to assess collinearity between variables, and as a result some variables that were collinear with other variables were excluded from the multivariable stage of the analysis.

In the early disease group, associations between anti-ApoA-1 levels and clinical outcomes were analysed using the statistical analysis software Prism. Univariable analysis was performed using the Mann–Whitney U test, as the sample did not follow a normal distribution. Statistical significance was considered when $P < 0.05$. Survival curves for cardiovascular disease events and mortality were produced using the Kaplan–Meier method. For the analysis of mortality, patients who were not known to have died

during the period of follow-up were censored at the end of the study period or at the time of loss to follow-up if that applied (for example, patients who moved away from London). For the analysis of CVD events, patients who had no such events were censored at the time of death, loss to follow-up or the end of the study period. The survival curves were compared using the log-rank test.

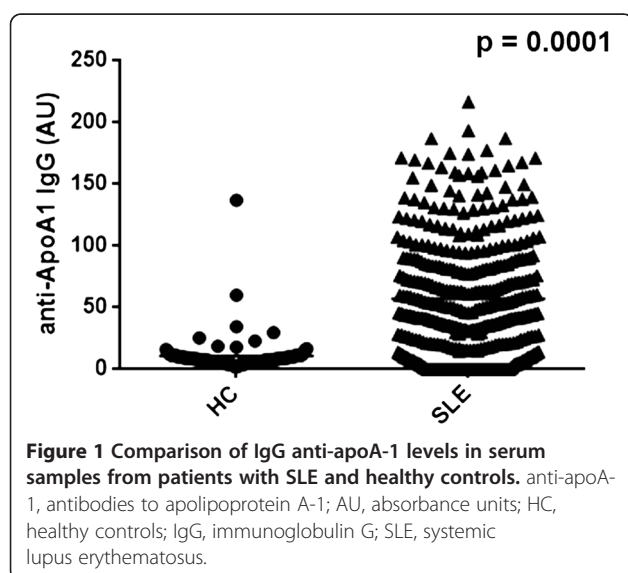
Results

Longitudinal group: elevated IgG anti-apoA-1 level was associated with high disease activity, high-dose steroids and not being treated with hydroxychloroquine

The mean age of the 49 patients in the longitudinal group was 36 years (SD 13.0) and 81% were female. Twenty-three patients were Caucasian, 18 were Afro-Caribbean and eight were other ethnicities. Twenty-one patients were anti-Ro-positive, six were anti-La-positive, 15 were anti-RNP-positive and 11 were anti-Sm-positive. During the follow-up period, 29 patients had at least one elevated anti-dsDNA level, 32 had at least one low C3 level and 46 suffered at least one flare (BILAG A or B in at least one system). Flares in all eight systems of the classic BILAG index were represented in the cohort.

Figure 1 shows that the IgG anti-apoA-1 level was higher in the 397 samples from patients with SLE (median 48.5, interquartile range (IQR) 16.0 to 87.5) than in the 100 healthy controls (median 8.0, IQR 5.9 to 10.7) ($P = 0.0001$). A positivity cutoff value was defined as the 97.5th percentile of 100 healthy controls (46.7 AU). Fifty per cent of the samples from patients with SLE were found to be IgG anti-ApoA-1-positive.

Table 1 presents the results of univariable analysis to assess association of 24 clinical, demographic and serological variables with serum IgG anti-apoA-1 level.



IgG anti-apoA-1 levels were significantly lower in patients with positive anti-La and anti-Sm but there was no relationship with C3 or anti-dsDNA.

Patients with persistent moderate/high disease activity had 30% higher IgG anti-apoA-1 levels than those with persistently low activity. Those with haematological BILAG scores of A or B on the day of the sample had higher IgG anti-apoA-1 levels than those with scores of C, D or E ($P < 0.001$). A similar trend was observed for patients with renal or cardiorespiratory A or B scores although statistical significance was not reached ($P = 0.06$ for both).

Patients treated with hydroxychloroquine had IgG anti-apoA-1 levels 30% lower than those who were not taking hydroxychloroquine, whereas those on higher dose prednisolone (>7.5 mg/day) had levels 39% higher than those taking lower doses.

Table 2 presents the results of multivariable analysis. No collinearity was observed between variables. Only negativity for anti-La and anti-Sm, haematological disease activity and taking higher dose prednisolone remained as independently associated variables.

As shown in Figure 2, IgG anti-apoA-1 levels varied over time and paralleled disease activity (measured by global BILAG score) closely in many (Figure 2A,B,C,D,E, F), but not all patients (Figure 2G,H).

IgG anti-apoA-1 positivity was seen in 27% of early disease samples and was not associated with mortality or CVD

The mean age of the 499 subjects at the time when the early disease sample was taken was 30 years (SD 12.27, range 1 to 77) and 91.5% were female. Ethnic distribution was Caucasian 61%, African/Caribbean 20.5%, South Asian 11.4%, East Asian 4.6%, and other 2.5%.

Median IgG anti-apoA-1 levels were significantly higher in patients with SLE (median 21.4 AU, IQR 11.2 to 52.9) than in healthy controls (median 8.0 AU, IQR 5.9 to 10.7) ($P < 0.05$) and 27% of patients tested positive in the first sample obtained after diagnosis of SLE. There was no correlation between the order of acquisition of samples and IgG anti-apoA-1 level (Spearman correlation coefficient -0.115), suggesting that storage time does not affect IgG anti-apoA-1 level.

As seen in the longitudinal group, there were no significant associations between IgG anti-apoA-1 level and age, gender or ethnicity.

During follow-up, 40 patients (8.0%) suffered a CVD event; that is, coronary heart disease confirmed by enzyme or electrocardiography changes of myocardial infarction or by angiography for nonmyocardial infarction CAD or ischaemic stroke confirmed by imaging. The prevalence of CVD events did not differ between anti-apoA-1-positive and anti-apoA-1-negative patients (6.7% vs. 8.5%, $P > 0.05$). A Kaplan–Meier survival curve showing

Table 1 Univariable analysis of factors associated with serum IgG anti-apoA-1 levels in the longitudinal group

Variable	Category	Ratio (95% CI)	P value
Gender ^a	Female (n = 336)	1	0.30
	Male (n = 61)	1.20 (0.85, 1.69)	
Disease duration	Ratio given per 5-year increase	1.00 (0.90, 1.11)	0.99
Age (at diagnosis)	Ratio given per 10-year increase	1.02 (0.88, 1.18)	0.81
Ethnicity ^a	Caucasian (n = 182)	1	0.46
	Afro-Caribbean (n = 146)	1.19 (0.90, 1.57)	
	Other (n = 69)	1.07 (0.75, 1.53)	
Any ENA ^b	No (n = 165)	1	0.13
	Yes (n = 232)	0.82 (0.63, 1.06)	
Anti-Ro ^b	No (n = 226)	1	0.41
	Yes (n = 171)	0.90 (0.69, 1.17)	
Anti-La ^b	No (n = 345)	1	0.002
	Yes (n = 52)	0.54 (0.36, 0.80)	
Anti-Sm ^b	No (n = 298)	1	0.004
	Yes (n = 99)	0.64 (0.47, 0.87)	
Anti-RNP ^b	No (n = 288)	1	0.46
	Yes (n = 109)	1.11 (0.84, 1.47)	
Anti-dsDNA level	<50 IU/ml (n = 177)	1	0.29
	≥50 IU/ml (n = 170)	1.13 (0.90, 1.43)	
C3 level	<0.9 g/l (n = 139)	1	0.09
	≥0.9 g/l (n = 208)	0.82 (0.66, 1.03)	
Disease activity in general system ^c	A, B (n = 31)	1	0.32
	C, D, E (n = 344)	0.84 (0.60, 1.18)	
Disease activity in mucocutaneous system ^c	A, B (n = 41)	1	0.48
	C, D, E (n = 334)	0.89 (0.65, 1.22)	
Disease activity in neuropsychiatric system ^c	A, B (n = 18)	1	0.77
	C, D, E (n = 357)	0.93 (0.57, 1.52)	
Disease activity in musculoskeletal system ^c	A, B (n = 47)	1	0.14
	C, D, E (n = 328)	0.80 (0.60, 1.08)	
Disease activity in cardiorespiratory system ^c	A, B (n = 13)	1	0.06
	C, D, E (n = 362)	0.66 (0.42, 1.02)	
Disease activity in vascular system ^c	A, B (n = 12)	1	0.40
	C, D, E (n = 363)	0.80 (0.47, 1.34)	
Disease activity in renal system ^c	A, B (n = 41)	1	0.06
	C, D, E (n = 329)	0.75 (0.55, 1.01)	
Disease activity in haematological system ^c	A, B (n = 95)	1	<0.001
	C, D, E (n = 280)	0.63 (0.50, 0.79)	
Overall disease activity over last four assessments	Persistently low (n = 166)	1	0.01
	Persistently moderate/high (n = 209)	1.30 (1.06, 1.59)	
Hydroxychloroquine ^d	No (n = 223)	1	0.003
	Yes (n = 174)	0.70 (0.55, 0.88)	
Immunosuppression ^d	No (n = 200)	1	0.35
	Yes (n = 197)	0.90 (0.73, 1.12)	
Oral prednisolone ^d	≤7.5 mg/day (n = 101)	1	

Table 1 Univariable analysis of factors associated with serum IgG anti-apoA-1 levels in the longitudinal group
(Continued)

	>7.5 mg/day (n = 296)	1.39 (1.13, 1.71)	0.002
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Bold data indicate values of $P < 0.05$. anti-apoA-1, antibodies to apolipoprotein A-1; BILAG, British Isles Lupus Assessment Group; CI, confidence interval; IgG, immunoglobulin G; ENA, antibodies to extractable nuclear antigens. ^aFor gender, n values refer to the numbers of samples taken from female and male subjects, rather than numbers of females and males in the cohort of patients. A similar stipulation applies to ethnicity, where n values refer to the number of samples taken from patients of each ethnic group. ^bFor ENA, anti-Ro, anti-La and anti-Sm, we did not have results from the date of every sample but it is assumed that positivity and negativity for these antigens generally remain stable. ^c'Disease activity in general system' refers to the BILAG score (A, B, C, D or E) in the General Category of the BILAG index on the day when each sample was taken. The same principle applies to all of the other organ systems listed in the table, which are the eight different categories recorded in the BILAG. ^dRefers to drugs being taken on the date of the sample.

the percentage of subjects in the anti-apoA-1-positive and anti-apoA-1-negative groups free of CVD at all time points up to 34 years of follow-up showed no significant difference between groups (Figure 3; $P = 0.89$ by log-rank test).

Similarly, positivity for IgG anti-apoA-1 did not predict onset of damage during follow-up. Table 3 presents damage scores at 5, 10, 15 and 20 years after stratification into quartiles based on the early disease sample IgG anti-apoA-1 level with no significant differences in damage score between the quartiles at any time point.

During follow-up, 63.0% and 24.0% of patients were at some time positive for anti-dsDNA and anti-cardiolipin antibodies respectively. One-quarter of the patients were found to be rheumatoid factor-positive and over one-half were positive for antibodies to extractable nuclear antigens (14.9% anti-Sm, 27.8% anti-RNP, 37.5% anti-Ro and 13.9% anti-La).

Patients who developed anti-dsDNA positivity had higher IgG anti-apoA-1 in their early disease samples than those who did not (median 22.5 AU vs. 17.1 AU, $P = 0.0012$) and a similar relationship was found for those who developed anti-cardiolipin antibodies (IgG anti-apoA-1 25.5 AU vs. 21.0 AU, $P = 0.025$). There were no relationships with other autoantibodies.

Regarding mortality, 13% of patients died ($n = 66$), 48 of them before the age of 60. A Kaplan–Meier survival curve showing the percentage survival of subjects in the anti-apoA-1-positive and anti-apoA-1-negative groups at all time points up to 34 years of follow-up showed no significant difference between groups (Figure 4; $P = 0.22$ by log-rank test).

There was no indication that deaths in patients younger than 60 or in patients with raised IgG anti-apoA-1

were due disproportionately to CVD. In the over 60s, the causes of death were 17% CVD, 44% cancer, 17% infection, 6% renal and 17% other. In the under 60s, the causes were 13% CVD, 17% cancer, 31% infection, 8% renal and 31% other. Of the six patients who died from CVD under the age of 60, only one had positive IgG anti-apoA-1 at the time of diagnosis.

Discussion

Previous studies confirmed the presence of IgG anti-apoA-1 antibodies in patients with SLE, indicated an association with higher disease activity and suggested a possible link with the development of CVD [1-4]. In this paper we have confirmed the association with disease activity by carrying out multivariable analysis of factors affecting IgG anti-apoA-1 levels in longitudinal samples from a group of 49 patients whose disease activity varied over time. We investigated the prevalence of elevated IgG anti-apoA-1 in early disease and its predictive value for CVD and mortality by studying 499 patients followed for a mean of 12.1 years after the date of the sample.

We showed that 27% of 499 patients were positive for IgG anti-apoA-1 very early in the disease course. Arbuckle and colleagues showed that many autoantibodies are present in patients with SLE even in stored samples taken before diagnosis of SLE [28]. However, anti-apoA-1 levels in early disease samples have not been studied before.

We found no association between IgG anti-apoA-1 positivity early in disease and increased risk of dying as survival curve analysis showed no difference between the anti-apoA-1-positive and anti-apoA-1-negative groups. Development of damage (due to disease activity or therapy) is a

Table 2 Multivariable analysis of factors associated with IgG anti-apoA-1 level in the longitudinal cohort

Variable	Category	Ratio (95% CI)	P value
Anti-La	No	1	0.006
	Yes	0.58 (0.39, 0.86)	
Anti-Sm	No	1	0.001
	Yes	0.58 (0.43, 0.80)	
Activity in the haematological system	A, B	1	<0.001
	C, D, E	0.66 (0.53, 0.83)	
Steroids	≤7.5 mg/day	1	0.008
	>7.5 mg/day	1.34 (1.08, 1.66)	

anti-apoA-1, antibodies to apolipoprotein A-1; CI, confidence interval; IgG, immunoglobulin G.

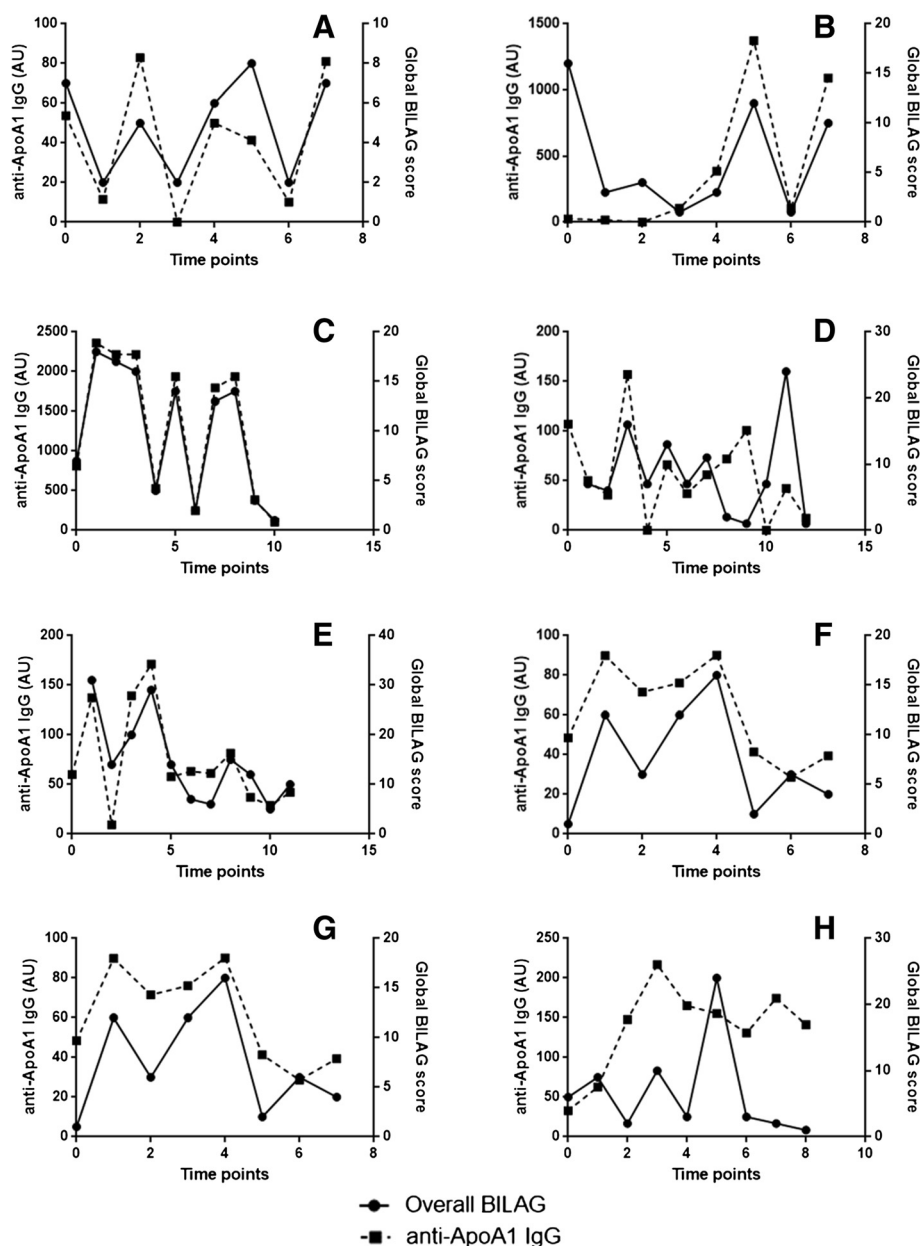
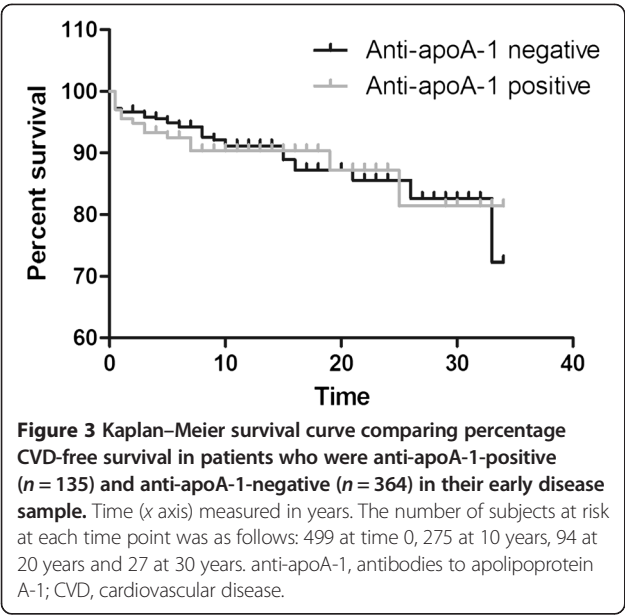


Figure 2 Longitudinal variation in IgG anti-apoA-1 levels and global BILAG score in eight patients with SLE over time. Time points on the x axis are successive follow-up visits. IgG anti-apoA-1 parallels global disease activity in many (A) to (F) but not all (G), (H) patients. anti-apoA-1, antibodies to apolipoprotein A-1; AU, absorbance units; BILAG, British Isles Lupus Assessment Group; IgG, immunoglobulin G.

powerful risk factor for mortality [29] in SLE but we found no association between IgG anti-apoA-1 level and SLICC-DI score over a 10-year follow-up period in over 200 patients (Table 3).

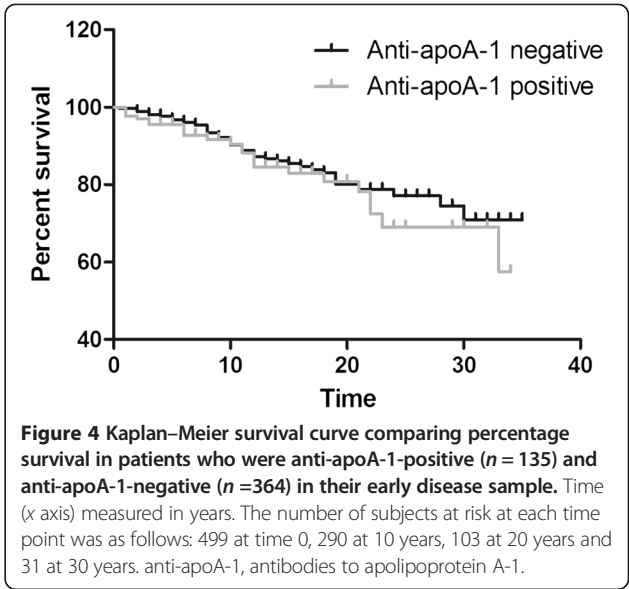
We found no association between IgG anti-apoA-1 positivity early in disease and development of CVD later. The numbers of patients with CVD were relatively small (40/499). We have thus found no convincing link between IgG anti-apoA-1 and CVD in patients with SLE in this study. This finding agrees with those of Radwan and

colleagues [4] in Egyptian patients with SLE but contrasts with those of Vuilleumier and colleagues, who found that, in 133 Swiss patients with RA followed for a median of 9 years [11], baseline IgG anti-apoA-1 positivity was strongly associated with risk of developing a major cardiovascular event (hazard ratio 4.7, 95% confidence interval 1.9 to 11.2), even after adjusting for standard CVD risk factors in multivariable analysis. However, this RA cohort was older (mean age 65 years), contained more males (30%) and had higher rate of CVD events compared



with our group or other SLE groups reported in the literature [1,4]. Longer-term studies may well be needed in SLE than in RA to identify predictive effects of factors such as autoantibodies on CVD risk. Notably, in a large inception cohort study, 1,249 patients with SLE were followed for a median of 8 years and only older age and male gender were identified as CVD risk factors in multivariable analysis [30].

Our study had a number of limitations. We did not study different isotypes of IgG anti-apoA-1 antibodies. In future, it may be worthwhile to investigate whether specific isotypes are more strongly associated with overall or organ-specific disease activity. We defined CVD by review of the notes and did not have detailed information on CVD events (such as troponin rise and degree of arterial stenosis). Other studies, such as those of Vuilleumier and colleagues defined CVD using harder endpoints [9,11]. The number of patients in the longitudinal group was relatively small at 49. The surprising finding that elevated IgG anti-apoA-1 levels were associated with negativity for anti-Sm and anti-La thus needs to be confirmed in a larger cohort. If this is confirmed, it may



be useful to investigate whether associations of IgG anti-apoA-1 and different forms of disease activity are different in anti-Sm-positive or anti-La-positive versus anti-Sm-negative or anti-La-negative patients.

Previously we showed that IgG anti-apoA-1 levels were higher in patients with persistently active disease than quiescent disease and rose at times of disease flare [2]. However, we did not study serial samples taken over long periods and were unable to carry out multivariable analysis. By studying samples taken from 49 patients longitudinally, we have confirmed the association of IgG anti-apoA-1 level with disease activity, particularly haematological activity. The association with haematological activity, however, may arise partly from the fact that there were more patients with BILAG A or B scores in the haematology system than in any other system. In previous papers we have also demonstrated associations between serological markers and activity in particular systems: anti-nucleosome antibodies and renal lupus [31], interleukin-6 and haematological lupus [32], and nitrated nucleosomes and vasculitis [33]. Hydroxychloroquine was associated with lower IgG anti-apoA-1, and high-dose corticosteroids with higher

Table 3 Mean damage scores over 20 years stratified by quartile of serum IgG anti-apoA-1 level in the early disease sample

	Lowest quartile	Second quartile	Third quartile	Highest quartile
Damage score at 1 year ($n = 236$)	0.14	0.13	0.10	0.08
Damage score at 5 years ($n = 236$)	0.63	0.43	0.45	0.42
Damage score at 10 years ($n = 209$)	1.06	0.60	0.96	0.65
Damage score at 15 years ($n = 131$)	1.23	0.81	1.33	0.97
Damage score at 20 years ($n = 69$)	1.33	1.06	1.88	1.00

anti-apoA-1, antibodies to apolipoprotein A-1; IgG, immunoglobulin G.

anti-apoA-1. In many patients, IgG anti-apoA-1 levels varied in parallel with disease activity over time. These results, however, do not prove a causal relationship between presence of IgG anti-apoA-1 antibodies and development of inflammation or disease activity in SLE. The possibility of a causal relationship would be an interesting question for future work.

Conclusion

In summary, this comprehensive analysis shows that IgG anti-apoA-1 antibodies are found commonly in patients with SLE and develop early in the disease course. Levels are associated with high disease activity and treatment with corticosteroids, but we have shown no convincing link with CVD. It remains possible that such a link will be shown in longer-term studies or by studying more sensitive measures of atherosclerosis such as plaque echogenicity, thickness, area or volume using vascular ultrasound scanning [34,35].

Abbreviations

anti-apoA-1: antibodies to apolipoprotein A-1; AU: absorbance units; BILAG: British Isles Lupus Assessment Group; CVD: cardiovascular disease; ELISA: enzyme-linked immunosorbent assay; IgG: immunoglobulin G; IQR: interquartile range; OD: optical density; RA: rheumatoid arthritis; SD: standard deviation; SLE: systemic lupus erythematosus; SLICC-DI: Systemic Lupus International Collaborative Clinics Damage Index.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

SCR designed the study, carried out the ELISA and statistical analysis, collected clinical data and helped draft the manuscript. MD carried out the ELISA and statistical analysis and revised the manuscript. PB carried out the multivariable statistical analysis for the data from the longitudinal group and revised the manuscript. KFA collected clinical data and revised the manuscript. OL collected clinical data and revised the manuscript. DI collected clinical data and revised the manuscript. IG collected clinical data and revised the manuscript. YI collected clinical data and revised the manuscript. SCH carried out the collection and analysis of damage scores. AR designed the study, collected clinical data, carried out the survival curve analysis and drafted the manuscript. All authors read and approved the final manuscript.

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